Mononuclear phagocyte activation and its role in HIV-1 pathogenesis

Thesis

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MONONUCLEAR PHAGOCYTE ACTIVATION AND ITS ROLE IN HIV-1 PATHOGENESIS

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ABSTRACT:

Three decades into the HIV-1 pandemic, it is increasingly apparent that generalized immune activation is a key pathogenic determinant of AIDS. Studies of immune activation, in the setting of HIV-1, have focused primarily on the adaptive immune response despite the role of monocytes and macrophages as key regulators of inflammatory responses. In this thesis, I focused on the relationships between mononuclear phagocyte activation and HIV-1 pathogenesis, both *in vitro* using model systems and *in vivo* in African patients with AIDS. Using a monocyte-derived-macrophage (MDM) model of M1 and M2a polarization, I found that activation transiently altered the capacity of macrophages to support productive HIV-1 infection and that the level of viral inhibition was dependant on the polarization phenotype. M1 and M2a polarized MDM also differed in the capacity to transmit virus to CD4+ T cells with M2a cells efficiently transmitting HIV-1 via a DC-SIGN dependant mechanism. In a cohort of treatment-naïve AIDS patients, *in vivo* activation of circulating monocytes and intestinal macrophages was strongly associated with HIV-1 pathogenesis. Monocyte activation in these patients was differentially regulated by HIV-1 and circulating LPS (marker of microbial translocation) and was associated with two clearly distinct activation profiles. At the tissue level, HIV-1 was associated with alterations in intestinal macrophage distribution, frequency and phenotype. In the colon, macrophage activation was negatively correlated with viral load and positively correlated with microbial translocation suggesting that, although inflammatory macrophages in the intestine may contribute to the control viral replication, they may also enhance microbial translocation and systemic activation. In summary, current findings add to current knowledge by showing that monocytes and macrophages are not only primary targets of HIV-1 but they also contribute to HIV-1 pathogenesis by playing a pivotal role in interactions occurring at the interface between immune activation, microbial translocation and the development of AIDS.

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ABBREVIATIONS:

3TC	Lamivudine
AICD	Activation Induced Cell Death
AIDS	Acquired Immune Deficiency Syndrome
APC	Allophycocyanin
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypepetide-like
ART	Anti-Retroviral Therapy
BFA	Brefeldin A
CA	Capsid protein, p24
CAF	Cell Antiviral Factor
CD	Cluster of Differentiation
$CD45RA^{+}CCR7^{+}$	Naïve T-cell
$CD45RA^{-}CCR7^{+}$	Central memory T cell (T_{CM})
$CD45RA^{-}CCR7^{-}$	Effector memory T cell (TEM)
$CD45RA^{+}CCR7^{-}$	Terminally differentiated effector T cell ($T_{EM}RA$)
CMV	Cytomegalovirus
CNS	Central Nervous System
CRF	Circulating Recombinant Form (of HIV-1)
CTL	Cytotoxic T-Lymphocyte
CXCR4 (X4)	CXC-Chemokine Receptor-4
CCR5 (R5)	CC-Chemokine Receptor-5
R5X4	CCR5/CXCR4-using (dual tropic) virus
CCMT	Comprehensive Care, Management and Treatment
CRF	Circulating Recombinant Forms
d4T	Stavudine
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific Intracellular adhesion molecule-3-Grabbing Non-integrin
D-MEM	Dulbecco modified Eagle's minimal essential medium
EDTA	Ethylene Diamine Tetra acetic Acid
EFV	Efavirenz
Env	HIV envelope
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
Gag	Group-specific antigen
GALT	Gut Associated Lymphoid Tissue
GIT	Gastrointestinal Tract
gp	glycoprotein
HAART	Highly Active Anti-Retroviral Therapy
HAD	HIV-associated dementia
HBV	Hepatitis B
HCV	Hepatitis C
HEV	High Endothelia Venules
HHV8	Human Herpes Virus 8
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HSV	Herpes Simplex Virus
HZV	Herpes Zoster Virus
IBD	Inflammatory Bowel Disease
IFN	Interferon

IL	Interleukin
IN	Integrase enzyme
IRIS	Immune Reconstitution Inflammatory Syndrome
IS	Immunological Synapse
JCV	John Cunning Virus
KS	Kaposi's Sarcoma
LAL	Limulus Amebocyte Lysate
LFA	Lymphocyte Function-associated antigen
LPS	Lipopolysaccharide
LTNP	Long-Term Non-Progressor
LTR	Long Terminal Repeat
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MA	Matrix protein, p17
mAb	monoclonal Antibody
MDDC	Monocytes-Derived-Dendritic Cell
MDM	Monocyte-Derived-Macrophage
MHC	Major Histocompatibility Complex
MP	Mononuclear Phagocyte
MPS	Mononuclear Phagocyte System
MTCT	Mother To Child Transmission
M-tropic	Macrophage tropic
MVB	Multi-Vesicular Body
NC	Nucleocapsid protein
Nef	Negative regulatory factor
NFκB	Nuclear Factor-κB
NHS	Normal Human Serum
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NSI	Non-syncytium inducing
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
OIs	Opportunistic infections
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PD1	Programmed death receptor
PE	Phycoerythrin
PE-CY5.5	Phycoerythrin cyanin 5.5
PE-CY7	Phycoerythrin cyanin 7
PFA	Paraformaldehyde
PI	Protease Inhibitor
PIC	Pre-Integration Complex
PMTCT	Prevention of Mother to Child Transmission
$Pr55^{Gag}$	Gag Polyprotein
R	Receptors
Rev	Regulator of Virus
RNA	RiboNucleic Acid
RNP	RiboNucleoProtein complex
RT	Reverse Transcriptase
SI	Syncytium Inducing
SIV	Simian Immunodeficiency Virus
Tat	Trans-activator of transcription

TB	*Mycobacterium Tuberculosis*
TCR	T Cell Receptor
T_H	T Helper
TLR	Toll Like Receptor
TNF-α	Tumor Necrosis Factor Alpha
TRAIL	TNF-related apoptosis-inducing ligand
TREM	Triggering Receptor Expressed on Myeloid cells
T-tropic	CD4+ T cell tropism
tRNA	transfer Ribonucleic Acid
V3	V3-loop of HIV-1 envelope glycoprotein, gp120
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
VL	Viral Load
VS	Virological Synapse
ZDV	Zidovudine

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CHAPTER 1
LITERATURE REVIEW

1.0 OVERVIEW OF THE HIV-1/AIDS PANDEMIC

1.0.1 Magnitude and Regional Differences

As the world enters its fourth decade of the acquired immunodeficiency syndrome (AIDS) pandemic, a cumulative total of more than 60 million people have been infected with the human immunodeficiency virus type 1 (HIV-1) (**Figure 1.1**). Of the approximately 33 million people currently living with HIV-1/AIDS, 30.8 million are adults and 2.2 million are children under the age of 15 (1). Advances in antiretroviral therapy (ART) have reduced transmission rates and provided clinical relief to many HIV-1-infected patients living in Europe, North America and other regions of the developed world but the costs and toxicities of these treatments are substantial (2). While ART is becoming more universally available, the HIV-1/AIDS epidemic continues to evolve and expand in some low- and middle-income countries. New infections continue to occur at a rate of 2.7 million per year and each year 2 million people in the developing world die of AIDS (1). The worst affected region, sub-Saharan Africa, currently accounts for 68% of persons living with HIV/AIDS worldwide and for 76% of all deaths due to AIDS (1). Eight countries in southern Africa have exceeded an overall prevalence rate of 15% and in South Africa alone, more than 5 million people are infected (1).

Perhaps one of the most perplexing aspects of HIV/AIDS has been the unequal worldwide spread of the virus. In the industrialized world, the HIV-1 epidemic was initially concentrated in high-risk populations (men who have sex with men, injecting drug users, commercial sex workers). Between 1981 and 2005, three quarters of newly reported infections in the United States were among men who have sex with men (3). In contrast, in sub-Saharan Africa the HIV-1 epidemic is more generalized. As of 2007, approximately 61% of infected Africans were women who acquired their HIV-1 through heterosexual intercourse (1). Unlike transmission pathways that are confined to specific high-risk groups, heterosexual transmission targets a large self-sustaining population. As a

consequence, the AIDS epidemic in the developing world is composed of many sub-epidemics characterized by different behavioral and biological properties.

1.0.2 The Virus: Its Origins and Salient Characteristics

HIV, the pathogen responsible for AIDS, is a member of the lentivirus family of retroviruses. Two distinct types of HIV, HIV-1 and HIV-2, have been shown to infect and cause disease in humans. Phylogenetic analyses have revealed the simian origins of both viruses and provided an understanding of the factors contributing to their cross-species transmission (4). Available evidence indicates that the simian counterparts of HIV-1 and HIV-2 were introduced into the human population on at least seven different occasions (4). HIV-2 appears to have been transmitted by sooty mangabeys, whereas chimpanzees are the most probable source HIV-1 (5). It is generally accepted that the main (M) group of HIV-1 viruses originated through a single cross-species transmission (4, 5) that occurred approximately 50-80 years ago (6, 7) in Central West Africa (8). While controversy still surrounds the exact mode of transmission to humans, the progenitors were probably passed to human hunters through blood-borne transmission (9). Unlike HIV-1 which is found globally, HIV-2 has a restricted distribution that is confined primarily to West Africa and India (10). HIV-2 is also associated with significantly slower progression to immune deficiency and lower transmission rates compared to HIV-1 (11).

HIV-1, the primary determinant driving the current AIDS pandemic, is a rapidly evolving RNA virus. Similar to other RNA viruses, such as hepatitis C (HCV), the ability of HIV-1 to mutate and escape immune detection is an integral part of the pathogenic process that results in viral persistence, uncontrolled transmission and the emergence of devastating pandemics (12). Despite their extensive functional and structural diversity, many RNA viruses, including HIV-1, share the following properties: 1) a small genome (3 to 30 kb) with relatively few genes; 2) high mutation rates due to an error prone reverse transcriptase (or replicase); 3) high production rates, in excess of 10^9 virions per days, and

4) long-term persistence of infection in large numbers of affected persons. These properties provide tremendous scope for the diversification, expansion and spread of a range of viral variants. In the case of HIV-1, a double-stranded RNA virus, the ability to generate escape mutants is further enhanced by frequent recombination between the two strands of RNA. This extreme plasticity, which is of unprecedented proportions in the case of HIV-1, influences the virus' immunogenicity, transmissibility and infectivity, and is a major impediment to the development of an efficacious AIDS vaccine (13).

1.0.3 HIV-1 Subtypes and their Geographic Distribution

As a result of a high evolutionary rate, HIV-1 group M, the viruses that dominate the global pandemic, have evolved into nine major subtypes (A-D, F-H, J and K) and 43 circulating inter-subtype recombinant forms (CRFs) (14-16). Within the A and F subtypes, separate sub-clusters have been given the designations A1, A2, A3 and A4, and F1 and F2 (16). Subtype B, the first recognized and most studied variant of HIV-1, continues to predominate in North America, Western and Central Europe, and Australia. This subtype accounts for only a small fraction of the worldwide pandemic (<15%) (16). On a global scale, the most prevalent and frequently transmitted subtypes are C, A (sub-subtype A1) and the A/G recombinant, CRF02_AG (**Figure 1.2**) (16). Subtype C viruses are responsible for the explosive outbreaks of HIV-1 in southern Africa, India and China. C/D recombinants are common in Tanzania, and B/C recombinants are prevalent among injecting drug users in China (17). Other subtypes are similarly restricted in their geographic distribution. Subtype D is limited primarily to East and Central Africa with sporadic cases being detected in Southern and Western Africa (18). Interestingly, E has never materialized alone, but rather appears as an A/E recombinant that is present in Asia and some parts of Africa (16). Subtypes H, J and K are relatively rare compared to other subtypes and are typically localized to isolated areas. Although these general classifications are still helpful, increasing immigration and globalization are having a

dramatic impact on the distribution of HIV-1 subtypes (18). As a result, ongoing attempts to organize and classify HIV-1's genetic variants based on phylogeny are becoming increasingly difficult (16). At present, >40% of all new infections in Europe are of the non-B African and Asian subtypes (16, 19).

1.0.4 Functional Implications of Subtype Diversity

The genetic variation within a given HIV-1 subtype typically ranges from 15% to 20%, whereas variation between subtypes is in the order of 25% to 35% (15). In contrast to HCV where there are clear-cut differences, not only in the geographic distribution of different viral subtypes but also in their transmission routes and response to therapy, the biological and functional significance of HIV-1 subtype diversity remains elusive.

The impact of subtype diversity on disease progression has been the subject of several international studies. Although the results have been somewhat discrepant, these studies have suggested there may be important differences between subtypes (20, 21). For example, a study of 54 female sex workers in Senegal found that women infected with subtypes C, D and G had increased rates of disease progression relative to those infected with subtype A (22). A larger study from Uganda found faster progression to death among patients infected with subtype D relative to A (23). Similarly, a Kenyan study found patients infected with subtype D had a higher mortality rate and a faster CD4+ decline relative to those with subtype A and C (24). It has also been suggested that this increased mortality may be due to the higher prevalence of dual-tropic isolates among subtype D viruses (25). While it is difficult to design a comparative study, preliminary data suggests that there may also be subtype-specific differences in transmission efficiency. A recent study of pregnant women in Kenya suggested that C viruses may be more transmissible than subtypes A and D due to increased shedding of HIV-1-infected cells in the vaginal mucosa (16). Another recent study found that subtype C (relative to subtype B) had higher

rates of mother to child transmission (MTCT) compared to non-C viruses (26). Although interesting, it is unclear if these findings are relevant on a global scale.

1.1 VIRUS STRUCTURE AND LIFE CYCLE

1.1.1 Virion Structure and Composition

Lentiviruses are particularly skillful at evading the host immune system. They are characterized by prolonged periods of disease progression, have high mutations rates, are transmitted exclusively by the exchange of body fluids and most importantly, cannot be easily eradicated from infected cells. Lentiviruses also differ from other viruses in their ability to infect non-dividing cells (27). At the structural level, HIV-1 virions are composed of a nucleoprotein core surrounded by a proteolipid envelope containing surface (gp120) and transmembrane (gp41) glycoproteins (28). The core of the virus contains an electron dense conical p24 capsid containing two copies of viral genomic RNA held together by RNA-binding proteins and multiple complementary nucleic acid regions (27). The viral enzymes (reverse transcriptase, RNase H and integrase), tRNA and the accessory proteins required for viral infectivity, cDNA synthesis and virion assembly during budding from the cell surface, are also found in the core of the viral particle (27).

In common with all viruses, the HIV-1 genome encodes three major genes; *gag, pol and env*. The *gag* gene encodes a $Pr55^{gag}$ precursor polyprotein which, when cleaved by HIV-1 protease, produces matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7), p6 and two spacer proteins, p1 and p2 (27). The viral enzymes are formed by cleavage of $Pr160^{gag\text{-}pol}$, a polyprotein derived by ribosomal frame-shifting (27). The envelope (Env) glycoproteins, gp120 and gp41, are also synthesized as a polyprotein precursor, gp160. However, unlike Gag and Pol, Env is processed by a cellular protease during trafficking to the cell surface, a process that results in the generation of the surface gp120 and trans-membrane gp 41 proteins (27). In addition to enzymes and structural proteins, HIV-1 also

encodes a number of regulatory and accessory proteins. Tat is a critical transcriptional activator of the LTR region (29), whereas Rev plays a major role in the transport of viral RNAs from the nucleus to the cytoplasm (30). Vpu, Vif, Vpr and Nef have been termed "accessory" or "auxiliary" proteins to reflect the fact that they are not uniformly required for virus replication. Interestingly Vpu promotes CD4 degradation, Vif overcomes the inhibitory effects of host cell factors (namely APOBEC), Vpr promotes the infection of macrophages and Nef promotes the down regulation of surface CD4 and class I MHC expression, blocks apoptosis and enhances virion infectivity (31).

1.1.2 Membrane Fusion and Virus Entry

Entry of lentiviruses (including HIV-1) into host cells, closely resembles that of other retroviruses and requires fusion of the viral envelope with the target cell membrane, a process which allows the inner contents of the viral particle to gain access to the cytoplasm (28). The first step in the entry process is initiated by the binding of HIV gp120 to CD4 on the surface of target cells (32, 33). This interaction induces conformational changes in the conserved core region of gp120, leading to unmasking of the chemokine co-receptor (CCR5 or CXCR4) binding site (28, 34). Interaction with these chemokine co-receptors promotes close apposition of the virus and cell membrane and results in further conformational changes in the gp120/gp41 complex, eventually activating gp41 (i.e. allowing its exposure and insertion into the target cell membrane) (35). The gp41 protein then experiences further structural re-organization into a six-helix bundle structure, a process which changes the free energy state of the protein and provides the force required for fusion, pore formation and entry of the viral capsid into the target cell (36, 37).

1.1.3 Tropism and Co-receptor Usage

HIV-1 isolates vary in their co-receptor usage, and can use either CXCR4 (X4) or CCR5 (R5) to enter cells. However, co-receptor use is not random and CCR5 is the main receptor used by the majority of isolates worldwide. Genetic differences in the V3 loop of gp120 are a major determinant of cellular tropism. Envelopes that interact with CCR5

typically have a low number of positively charged V3 amino acids, whereas CXCR4-using envelopes usually contain V3 sequences with an excess of positively charged amino acid residues, most notably at positions 11 and/or 25 (38-41). Interestingly, R5X4 isolates have a V3 charge pattern that is similar to X4 envelopes. The dual tropic properties of these viruses are complex and involve multiple discontinuous regions of Env (42-44).

Historically, HIV-1 has been classified by its *in vitro* infection profile as either monocyte-derived macrophage tropic (M-tropic)/non-syncytium inducing (NSI) or as T-cell line tropic (T-tropic)/syncytium inducing (SI). M-tropic/NSI variants predominantly use CCR5 for entry into macrophages and have been designated R5 viruses, whereas T-tropic/SI variants predominantly use CXCR4 for entry into T-cell lines and have been designated as X4 viruses (45). Dual tropic R5X4 viruses can infect both macrophages and T-cells. This classification, however, is not completely accurate, as primary CD4+ T cells can be infected by R5 viruses and some macrophages can be infected by some primary X4 isolates (46, 47). In reality, the determinants of co-receptor usage are likely to be complex and involve parameters of viral replication, host genetics and immune activation, in addition to genetic changes in the HIV-1 envelope gene (48, 49).

Interestingly, R5/NSI viruses are preferentially transmitted to a new host, even when the donor is infected with both R5 and X4 phenotypes (50). This phenomenon may reflect the fact that macrophages are particularly abundant in mucosal membranes and that they are less susceptible to CTL responses (51). As a result, R5 viruses can escape the human immune system more efficiently than X4 viruses (51). In approximately 40-50% of patients infected with subtype B, progression from asymptomatic to late-stage disease is associated with a switch from R5 to X4, or R5X4 usage (52, 53). This switch in co-receptor usage usually takes about 7 years to occur, involves amino acid changes in V3 and is associated with a more rapid decline in CD4 T-cells and progression to AIDS. The reason for the enhanced cytopathic effect of X4 viruses is not known, but may be due to

altered replication kinetics and/or the ability of X4 strains to infect naïve $CD4^+$ T-cells in addition of $CD^+CD45RO^+$ memory T-cells (54, 55). Accumulated evidence suggests the conversion to an X4/SI phenotype occurs only once during the course of HIV-1 infection, that the transition occurs abruptly and that R5X4 viruses may represent an intermediate in the evolution between R5 and X4 viruses (56).

1.1.4 Uncoating and Viral Integration

HIV-1 replication begins soon after fusion with the plasma membrane and the release of the viral core into the cytoplasm of the target cell (**Figure 1.3**). During this process, the viral capsid (CA) disassembles releasing the viral ribonucleoprotein complex (RNP) (57, 58). This process, known as "uncoating", is critical for reverse transcription and synthesis of the double-stranded viral DNA intermediate, as well as the formation of a functional pre-integration complex (PIC). In the PIC, the reverse-transcribed HIV-1 DNA is present in a high molecular-weight nucleoprotein complex containing viral proteins [integrase (IN), matrix protein (MA), reverse transcriptase (RT), nucleocapsid (NC), and Vpr] (59-61). Although the structure of the PIC has not been well defined, it has been suggested that the ends of the viral DNA are "tethered" by an assembly of proteins that protect and prime the DNA for integration (62, 63). After entering the nucleus, integration into the host genome is directed by cellular components of the PIC such as mobility group protein HMG 1(Y) (64, 65). Insertion of the viral genome occurs in genes that are highly transcribed by RNA Pol II. This process is catalyzed by viral IN and involves both terminal cleavage and ligation reactions (66, 67)

The ability of HIV-1 and other lentiviruses to infect non-dividing cells suggests that their PIC can be actively transported into the nucleus. Current knowledge indicates that nuclear import of the HIV-1 PIC is an ATP-dependent process involving a nuclear transport pathway and a viral nuclear localization signal (68). Other factors that may be important for nuclear targeting of the PIC include the karyophilic IN, MA and Vpr proteins

(69, 70), as well as a proviral structure known as the central DNA flap (71-73). Some studies have suggested that CA protein may also play a role in the infection of non-dividing cells, by serving as a link between uncoating and nuclear import of the PIC (74).

1.1.5 Viral Replication and Transcription

The transcription of HIV-1 proviral DNA is profoundly affected by the state of host cell activation and is regulated by interactions between cellular transcription factors and the HIV-1 5'-long terminal repeat (LTR) (75). This is due to the fact that a number of the sequences present in the viral LTR closely resemble the promoter regions present in the human genome. As a result of these similarities, HIV-1 is able to utilize the transcription machinery of the host for viral gene expression and replication. The HIV-1 promoter is divided into 3 distinct regions (76, 77). Two of these, the modulatory enhancer and core promoter regions, are located upstream of the transcription start site. The third region, the Trans-Activation Response region (TAR) lies downstream of the start point. The core promoter region interacts with SP1 and TATA binding proteins to mediate a basal level of transcription (78). Enhancement of basal transcription can occur, either through interactions between the virus-encoded Tat protein and TAR region of the viral LTR, or through the binding of host transcriptional factors to the modulatory enhancer region (76, 77). The enhancer region contains at least 6 elements that bind different cellular factors, the most studied being Nuclear-Factor-κB (NFκB) (75). The regulation of viral transcription in cells of monocyte-macrophage lineage appears to be more complex than in T cells. In monocytes and macrophages cellular activation can differentially affect viral transcription depending on state of cellular differentiation (monocyte vs. macrophage) (79-81). IL-4 and IL-13, for example, enhance viral replication in monocytes, but inhibit replication in differentiated monocyte-derived macrophages (MDM) (260). Furthermore, pro-inflammatory and immune modulating cytokines such as TNF-α, as well as gene products of DNA viruses, can induce (or suppress) or HIV-1 replication at both the

transcriptional and post-transcriptional levels depending on whether stimulation occurs before or after infection (78, 82-84).

1.1.6 Assembly and Maturation of HIV-1 Virions

The Gag polyprotein, $Pr55^{Gag}$, directs the recruitment of all components required for the efficient assembly and release of HIV-1 virions (85, 86). All four domains of $Pr55^{Gag}$, matrix (MA), capsid (CA), nucleocapsid (NC) and p6, play an important role in this process (87-90). The N-terminal myristic acid moiety of MA interacts with acid phospholipids on the inner leaflet of the plasma membrane (89, 91); CA and NC promote Gag-Gag interactions and the p6 domain of Gag stimulates virus release by interacting with the endosomal sorting machinery (92, 93).

Although substantial progress has been made in understanding the viral and cellular factors that are necessary for $Pr55^{Gag}$ membrane binding, multi-merization and virion release, the intracellular site of HIV-1 assembly and the pathway of Gag-mediated trafficking to the assembly site still remain controversial. Some studies have reported that HIV-1 assembly occurs at the plasma membrane (94-96) whereas others have suggested that virus assembly may take place in an endosomal compartment and that release of the viral particles follows an exosomal pathway in which virus-containing endosomes fuse with the plasma membrane releasing their contents (97-99). The endosomal model of virus assembly has been of particular interest in the setting of HIV-1-infected macrophages. Studies conducted in monocyte-derived macrophages (MDM) suggest that these cells synthesize and accumulate virions in intracellular vacuoles that have the characteristics of late endosomes or multivesicular bodies (MVB) [ie. they express markers for MHC II, CD82 and the tetraspanins CD9, CD53, CD63 and CD81] that form part of large and complex invaginated plasma membrane domain (100-104). HIV-1 particles stored in this intracellular domain are protected from effector elements of the host immune system. Other studies have shown these archived viruses remain infectious for prolonged periods (ie. > 6 weeks) and can be subsequently transmitted across a transient adhesive contact, or

Virological Synapse (VS) (105). The VS is a multi-molecular complex that forms at the interface between an HIV-1-infected macrophage (or dendritic cell) and an uninfected CD4+ T cells (106-108). Formation of the synapse is driven by the interaction of HIV-1 gp120 on the donor cell and CD4/CCR5 or CD4/CXCR4 on the recipient cell and is stabilized by cell-cell adhesion interactions (109).

1.2 HIV IMMUNOPATHOGENESIS

1.2.1 Changing Concepts in HIV-1 Pathogenesis

Until recently, our understanding of HIV-1 pathogenesis was based primarily on changes in HIV-1 viral load and CD4+ T cell numbers in the peripheral blood of patients infected with subtype B virus (110, 111). In this traditional view, acute HIV-1 infection was accompanied by a burst of plasma viremia and a transient but often unimpressive decrease in CD4+ T cell counts. This was followed by a prolonged period of chronic infection that typically lasts for 10 years. During this time there was a gradual but profound loss of peripheral blood CD4+ T-cells (an average of 100 cells per μL of blood per year) leading, ultimately, to collapse of the immune system. This prolonged disease course, combined with studies showing high levels of HIV-1 replication in CD4+ T cells in lymph nodes, led to the "tap-drain" model of pathogenesis. In this model, CD4+ T cells that were killed by the virus were continuously replaced by new cells generated in the thymus, or through the homeostatic proliferation of peripheral CD4+ T cell pools (112, 113). Based on this model, progression to AIDS was attributed to a negative net balance in cell death and replacement.

However, the question of whether HIV-1, on its own, could cause such a massive loss of CD4+ T cells remained controversial. The main unresolved question related to whether HIV-1 replication directly killed CD4+ T cells or whether it acted indirectly by inducing CD4+ T cell activation, with cell death being a normal consequence of this process. Arguments against a direct cytolytic mechanism are that: 1) the number of

productively infected CD4+ T cells in peripheral blood is very low (0.01-1%); 2) HIV-1-mediated destruction of CD4+ T cells rarely occurs *in vivo* and 3) high levels of SIV replication in sooty mangabeys does not lead to CD4+ T cell depletion and AIDS (114). Collectively, these data suggest that factors other than virus-induced cytolysis must contribute to the high rates of CD4+ T cell death.

As a result of above ambiguities, investigators began to focus their attention on immune activation as the cause rather than the consequence of CD4+ T cell death. This view is supported by *in vitro* studies showing that cytokines can enhance viral replication and that chronic infection is associated with increased T cell turnover, increased numbers of activated T and B cells and increased levels of pro-inflammatory cytokines (112, 115). However, as with the virus-driven model, the immune-activation theory is based almost exclusively on the analysis of peripheral blood, a compartment that contains only 2% of the body's total viral burden and a minority of its CD4+ T cells (114). Moreover, as we now know, blood is not reflective of the dynamic events occurring in lymphoid and mucosal tissues. The immune theory also does not explain why activation causes severe CD4+ T cell depletion while sparing activated CD8+ cells (112).

Recent studies have provided critical new insights into the mechanisms driving HIV-1 pathogenesis, as well as the timing and site of CD4+ depletion. These studies have revealed that the bulk of the CD4+ T cell depletion occurs during the first few weeks of infection and is localized primarily to the gastrointestinal tract (GIT) (**Figure 1.4**), a unique immune environment that contains most of the body's lymphoid tissue (gut associated lymphoid tissue (GALT)) (112). Studies of acute SIV infection in rhesus macaques, and of HIV-1 infection in humans, revealed that within 24 h of vaginal exposure, virus production was detectable in the GALT (116). This was followed, on days 5 to 10, by an intense burst of viral replication and a massive 70-90% depletion of GALT's memory CD4+CCR5 T cells (116, 117). In acute infection, the early loss of CD4+ T cells appears to be a direct consequence of CD4+ target cell infection (118, 119) and virus

induced Fas-mediated apoptosis (120). Based on these findings, it was proposed that this massive destruction of CD4+ T cells in the GALT may lead to an early breach in mucosal immunity, setting the stage for chronic progressive disease and further immunological deterioration. It was also suggested that the level of immune activation and the rate of progression to AIDS might be determined by the extent of CD4+ T cell depletion in the GALT during acute infection (112). Inherent in the GIT model, is the concept that injury to the immune component of the gut and the epithelial microenvironment may lead to enhanced translocation of luminal pathogens and microbial products, systemic immune activation and the exacerbation of HIV-1 infection (121). The GALT serves as a constant source of virus and because of its constant exposure to bacterial, viral and parasitic pathogens, continues to play a key role in the recruitment, activation and infection of new target cells throughout the course of HIV-1/AIDS (113).

In addition to CD4+ T cell depletion and chronic immune activation, other parameters such as immune exhaustion and a limited regenerative capacity may also contribute to AIDS pathogenesis. A model linking these three facets of HIV-1 disease – massive CD4+ T cell depletion, exhaustion of T cell regenerative capacity and paradoxical immune activation – was recently proposed by Appay and Sauce (122).

1.2.2 Mechanisms of HIV-1 Pathogenesis

a) CD4+ T cell Depletion

The main targets of HIV-1 during both acute and chronic infection are activated CCR5+CD4+ T cells that reside in lymph nodes and mucosal tissues, including the GIT (116, 123-125). However, the reason(s) for the persistent decline in the functionality and number of CD4+ T cells during the chronic phase of infection has been one of the most debated topics in AIDS research. While viral replication and cytolysis may play a key role in CD4+ T cell depletion during acute infection, other factors such as a limited regenerative capacity may be of greater importance during the chronic phase of HIV-1/AIDS (126).

Immune activation accelerates both the production and destruction of CD4+ T cells and poses a major burden on T cell renewal at the level of progenitor cells and thymic differentiation (114, 127-129). HIV-1 can also interfere with CD4+ T cell renewal through direct infection of thymocytes and by inducing collagen deposition in the thymus, damaging the thymic architecture and preventing normal T cell homeostasis (130, 131).

Another important pathway of CD4+ T cell depletion is programmed cell death (PCD) triggered by cytokines or HIV-1 proteins. Three distinct but overlapping PCD pathways have been described based on differences in the stimuli, the cytokine environment and the death-inducing signaling cascades. Two pathways, apoptosis and autophagy, have no affect the integrity of the plasma membrane and thus, do not induce an inflammatory response (114). The third pathway, necrosis, involves mechanical rupture of the membrane, the release of cellular organelles and the induction of a phagocytic inflammatory response (114). Autophagy and necrosis have been studied *in vitro*, while apoptosis has been extensively investigated both *in vitro* and in HIV-1-infected patients (114).

The majority of CD4+ T cells undergoing cell death in peripheral blood and lymph nodes are uninfected bystander cells that have a $CD45RO^{+}HLA\text{-}DR^{+}CD28^{-}$ phenotype and are killed by activation-induced cell death (AICD) resulting from repeated antigenic stimulation (132, 133). Although this process is predominately driven by the interaction of Fas and its ligand, FasL (134), it can also be triggered by interactions with the T Cell Receptor (TCR) in the absence of a second signal (co-stimulatory molecules) (135). Over-production of type-2 cytokines (IL-4 and IL-10) can increase susceptibility to AICD (114, 136). Interferon-α (IFN-α) produced in HIV-1-infected dendritic cells (DC) can also induce CD4+ T cell apoptosis through the TRAIL/Death Receptor-5 pathway (137).

In addition, HIV-1 proteins (Env, Tat, Vpr, Nef) can activate apoptotic cell death in bystander CD4+ T cells through caspase-dependent extrinsic and (or) intrinsic pathways. The extrinsic pathway involves members of the TNF family of proteins (TNF-R1, Fas,

Trail-R1 and Trail-R2) while, the intrinsic pathway is regulated by the balance between anti- and pro-apoptotic members of the Bcl-2 protein family. Interestingly, the binding of R5 tropic Env to CCR5 on target cells triggers the extrinsic apoptotic pathway, while the binding of X4 tropic Env to CXCR4 activates the intrinsic pathway, inducing mitochondrial transmembrane depolarization, release of cytochrome-c from the mitochondria into the cytosol, and activation of caspases-9 and -3 (138-140). Uptake of extracellular Tat via clathrin-mediated endocytosis (141), activates the intrinsic pathway (142, 143). Tat also upregulates Fas/FasL on T cells (144) and TRAIL expression on macrophages (145). Vpr induces apoptosis via a direct effect on mitochondrial permeability (146), whereas Nef induces apoptosis through CXCR4 (147).

b) CD8+ T Cell Exhaustion

HIV-1-specific CD8+ T lymphocytes are expanded during acute infection and play an important role in clearing the infection (148) and determining the viral set point (149, 150). The earliest and most effective CTL responses are directed primarily against the regulatory proteins, Tat and Rev (150-152). The CTL response is initiated by an interaction between the TCR on the CD8+ T cell and a peptide-MHC class I complex on an infected target cell. Although this interaction leads to both cytolytic (cell-mediated) and non-cytolytic (cytokine/chemokine) responses, these responses are only partially effective in controlling viral replication and spread. As would be expected, long-term non-progressors (LTNP) typically exhibit a stronger more sustained CTL response and a more durable suppression of HIV-1 viral load compared to patients with progressive disease (153).

The cytolytic response induced in HIV-1-specific CD8+ T cells involves the secretion of granzymes and perforins and the induction of Fas-FasL interactions. Secretion of the lytic granules occurs after activation of the TCR and involves their polarized transport and release at an immunological synapse (IS) formed between the CTL and the infected target cell (154). The lytic molecules released during this process cause the formation of pores in the target cell membrane, activating the caspase cascade and the

onset of apoptosis (154). Target cell apoptosis can also be triggered through a TCR/peptide-MHC-mediated upregulation of the Fas ligand (Fas-L) on the surface of the CD8+ T cell (150). Subsequent interaction of these Fas ligands with the Fas receptor on the infected target cell activates the caspase cascade of cell death (155, 156).

Non-cytolytic CD8+ T cell responses are mediated through the secretion of soluble factors such as β-chemokines (CCL3, CCL4 and CCL5), cytokines (including IFN-γ, TNF-α and IL-2) and the CD8+ T Cell Antiviral Factor, CAF. β-chemokines inhibit the entry of R5 viruses by binding to their natural ligand, CCR5 (157, 158). IFN-γ upregulates MHC class I expression and antigen processing (150, 159, 160), TNF-α activates apoptosis (160) and interleukin-2 (IL-2) induces the proliferation of CD8+ memory T-cells (150, 161). Soluble "CD8 antiviral factor (CAF)", a 27k, heat-stable soluble factor that has not yet been biochemically characterized, is a potent inhibitor of HIV-1 LTR-driven transcription (162-164).

However, the majority of HIV-1-infected patients are unable to maintain a durable control over viral replication due to rapid genetic evolution of the viral genome and the early generation of CTL and neutralizing antibody escape mutants (165-168). During the chronic phase of infection, CD8+ T cell dysfunction is closely linked to viral replication and is characterized by anergy and CD8+ T cell exhaustion. Anergy can be caused by quantitative changes that alter the HIV-1-specific response including increased CD8+ T cell apoptosis (via Fas-FasL), the loss of CD4+ T helper responses and (or) an increase in the breadth of the CD8+ response and an associated loss in the reactivity against early HIV-1 genes (150). Qualitative changes in the CD8+ CTL response during chronic infection include defects in IFN-γ and perforin secretion, as well as a loss of proliferating IL-2- and IFN-γ-secreting CD8+ T cell subsets (162, 169, 170).

Other factors that may contribute to the functional impairment of CD8+ T cells include Nef-mediated the down-regulation of MHC class I molecules on HIV-1-infected cells (171), the down-modulation of CD3-ζ on CD8+ T cells (172) and differences in the stage of cell differentiation (150). Although the mechanisms are not known, HIV-1 infection may also affect the differentiation of CD8+ T cells into memory subsets (122, 173, 174). At least four subsets of T cells have been proposed based on surface marker expression: naïve $CD45RA^{+}CCR7^{+}$; central memory, $CD45RA^{-}CCR7^{+}$ (T_{CM}), effector memory $CD45RA^{-}CCR7^{-}$ (T_{EM}) and terminally differentiated effector $CD45RA^{+}CCR7^{-}$ ($T_{EM}RA$) cells. The T_{CM} sub-population differentiates into cells that home to secondary lymphoid tissues, while T_{EM} cells produce high levels of perforin, IFN-γ and IL-4 in response to antigenic stimulation. $T_{EM}RA$ cells also produce high levels perforin and, together with a subset of $CD45RA^{+}$ T_{EM} cells, are known as rapid effectors. Infection with HIV-1 has been shown to skew CD8+ T cell differentiation toward a disproportionate increase in T_{EM} cells with reduced proliferative capacity and reduced perforin expression (150). These changes result in a major impairment/exhaustion of CD8+ T cell antiviral activity.

Other studies have suggested the up-regulation of PD-1 may mediate HIV-1-specific CD8+ T cell exhaustion and that blocking of the PD-1/PDL-1 pathway may represent a new therapeutic strategy (175-177). This pathway is comprised of the PD-1 receptor and its two ligands, PD-L1 and PD-L2, which are part of the B7-CD28 family. PD-1, originally described during chronic lymphocytic choriomeningitis virus (LCMV) infection, is highly expressed on exhausted T cells during chronic infection and its engagement by PD-L1 and PD-L2 inhibits immune responses (**Figure 1.5**) (177). PD-1 expression is elevated on circulating HIV-specific CD8+ T cells and the level of expression correlates with increased plasma viral loads and declining CD4+ T cell counts (178). Interestingly, *in vitro* blocking of the PD-1-PD-L1 interactions in patient PBMC leads to increased T cell proliferation and effector cytokine production (176-178). PD-1

expression is down-regulated in HIV-1-infected patients who exhibit a successful response to antiretroviral therapy (178). Further characterization of PD-1/PD-L interactions at the tissue level will be critical to understand the contribution of this pathway to HIV-1 pathogenesis.

1.2.3 Characteristics of Pathogenic versus Non-Pathogenic Infection

Additional support for an immune-based theory of disease progression comes from studies of non-human primates naturally infected with the closely related simian immunodeficiency virus, SIV. In contrast to Rhesus macaques which display high levels of immune activation, severe CD4+ T cell depletion and rapid SIV-associated disease progression, natural (non-pathogenic) SIV infection in sooty mangabeys and African green monkeys is characterized by sustained preservation of peripheral CD4+ T cells counts and a lack of disease progression despite sustained high levels of plasma viremia that are equivalent to, or greater than, those observed in HIV-1-infected persons with disease progression (179-182). Other characteristic features of non-pathogenic SIV include low levels of CD4+ and CD8+ T cell activation and proliferation in both blood and lymph nodes (182-184), low levels of T cell apoptosis (184-186), preserved regenerative functions in bone marrow, thymus and lymph nodes (187) and little or no cell-cycle dysregulation (179, 188, 189). Interestingly, non-pathogenic (natural) SIV infection is also associated with lower levels of peak immune activation and proliferation during primary infection and a more pronounced post-peak decline in immune activation (**Figure 1.6**) (179, 190, 191). Another interesting observation comes from studies of human immunodeficiency virus type 2 (HIV-2). Most persons infected with HIV-2, a less pathogenic strain of HIV, exhibit a relatively slow course of disease progression in association with low viral loads and levels of immune activation (192). These data provide compelling circumstantial evidence for a direct link between immune activation and retroviral disease progression.

1.3 INNATE IMMUNITY AND HIV-1 PATHOGENESIS

1.3.1 Role of Dendritic Cells (DC) in HIV-1 Transmission and Dissemination

DC are professional antigen presenting cells that play a key role in adaptive immune responses. They are a heterogeneous population of cells that exist in different functional states. Immature DC are antigen-capturing cells that reside in mucosal membranes. They have a high phagocytic and endocytic capacity, and continually sample the environment for evidence of foreign antigens and invasive pathogens (193, 194). Distinct subsets of mucosal DC express different pattern recognition receptors, such as Toll-like receptors, C-type lectins and scavenger receptors that are involved in antigen capture and the induction of immune responses (193). Depending on the receptor, these responses can involve cell signaling, increased expression of adhesion molecules, DC maturation and migration, and enhanced pathogen phagocytosis. Following activation of Toll-like receptors, DC mature into antigen presenting cells and migrate from peripheral tissues to secondary lymphoid sites where they stimulate naïve CD4+ T cells and CD8+ T cells, inducing them to develop into pathogen-specific helper and cytotoxic T cells, respectively (193-195).

HIV-1 has evolved a number of ways to exploit the biological properties of DC to facilitate virus transmission and dissemination, and evade antiviral immunity (196). Immature DC in mucosal membranes are among the first cells to be exposed to HIV-1 during heterosexual, homosexual and perinatal transmission. These DC are highly efficient at capturing and transporting HIV-1 virions to distal lymphoid tissues for transfer to susceptible CD4+ T cell targets (197). A number of studies have shown that DC-mediated transmission involves the capture of extracellular virions by DC-SIGN followed by subsequent transfer of the virus to permissive CD4+ T cells (**Figure 1.7**) (197-200). Some studies have suggested that DC-captured virions can also be internalized and stored in intracellular vesicles where they remain infectious for several hours (198, 201, 202). Although the exact nature of this storage compartment remains to be defined, it appears to

be a low pH non-lysosomal compartment that lacks early and late endosomal markers (197, 202, 203) but expresses markers that are characteristic of multivesicular bodies (MVB), namely, tetraspanins CD81 and CD9 (197, 204).

Although C-type lectins DC-SIGN (209), Langerhin (CD207), Mannose Receptor (CD206) and syndecans (heparin sulfate proteoglycans) can bind HIV-1, the ability to capture and *trans*-transmit virus to CD4+ T cells has, thus far, been demonstrated only for DC-SIGN (194, 197, 198, 205). Unlike classical interactions with entry receptors/co-receptors (CD4/CCR5 or CD4/CXCR4), the binding of HIV-1 to DC-SIGN does not lead to membrane fusion. Instead, DC-SIGN appears to function primarily as an attachment factor that facilitates the *trans*-transmission of HIV-1. At least one recent study has suggested that internalization is not essential for transmission and that DC-SIGN-mediated transfer of HIV-1 to T cells can occur with, or without, virus internalization (198, 200, 206). However, there are some controversies relating to the importance of DC-SIGN in the transmission process (205, 207). Most of this controversy arises from differences in the size of the viral inoculum used in tissue culture experiments. It now appears that DC-SIGN-mediated transmission of HIV-1 is most effective at low viral concentration and that high levels of CD4+ co-expression on DC-SIGN+ donor cells can lead to internalization and inefficient *trans*-transmission to T cells (for more details, see Chapter 4 in this thesis) (208).

Although the mechanisms underlying DC-mediated transmission of HIV-1 of T cells are not fully understood, the process has been shown to involve the formation of a tight intercellular junction or "Virological Synapse" (VS) between DC and CD4+ T cells (200). This junction is similar to an "Immunological Synapse" and is most likely initiated by a transient contact formed between a DC and T cell without a requirement for antigen specificity (200). Before DC-T cell contact, HIV-1 is evenly distributed inside the DC. However, within minutes after the initiation of cell-cell contact, viral particles concentrate at the site of contact on the donor (DC) side of the interface (209). The HIV-1 receptor/co-

receptors (CD4, CCR5 and/or CXCR4), and the adhesion molecule LFA-1 are recruited to the contact site on the recipient (T cell) side of the synapse (196, 200). This concentration of HIV-1 and its receptors/co-receptors at DC-T cell interface, combined with the directed release of HIV-1 virions to this site, may help explain why cell-mediated transmission of HIV-1 is several times more efficient than infection with cell-free viral particles (196, 209).

In addition, some strains of HIV-1 may be able to infect distinct subsets of langerhans, myeloid and plasmacytoid DC through a classical CD4/co-receptor-mediated entry mechanism (210, 211). However, this process is not particularly robust and seems to require a high viral input (212). Further studies are needed to determine whether the HIV-1 virions synthesized in DC accumulate in MVB and whether MVB-archived virion promote the long-term transmission of HIV-1 to T cells and serve as reservoirs of treatment-refractory virus (211, 213).

1.3.2 Infection and Dysfunction of Mononuclear Phagocytes

Blood monocytes are the progenitors of all macrophage (and DC) subsets including microglial cells, Kupffer cells and osteoclasts (214). Monocytes originate in the bone marrow from a common myeloid progenitor that enters the peripheral blood and circulates for a few days before migrating into peripheral tissues to differentiate into mature macrophages (215, 216). Some tissue macrophages, such as those in the lamina propria of the GIT, are thought to survive for few weeks before undergoing programmed cell death (215, 217). Such cells are continually replenished by newly recruited blood monocytes. Cells recruited to sites of inflammation typically have short half-life and exert potent pro-inflammatory effects. Other macrophages appear to have more prolonged half-lives ranging from several months (alveolar macrophages) to decades (microgial cells) (218) and may be renewed, at least in part, by local proliferation (219, 220).

Circulating monocytes are a heterogeneous population of cells that can phagocytose foreign material, present antigens to T cells and produce immunologically

important cytokines in response to LPS stimulation through the CD14/Toll-like receptor 4 (TLR-4) complexes (221-224). LPS-induced ligation of the CD14/TLR4+ receptor leads to signal transduction, mobilization of transcription factors and enhanced expression of tumor necrosis factor-α (TNF-α), a cytokine that induces cell proliferation and migration. Although CD14+ monocytes bind and support HIV-1 entry, accumulated evidence suggests that viral replication is restricted at, or prior to, reverse transcription and integration (225, 226) with some studies showing a block in virus entry that may be related to low CCR5 expression (226, 227). Other studies have suggested that the presence of with low molecular mass APOBEC3G may also contribute to the low susceptibility of CD14+ monocytes to HIV-1 infection (228).

Increasing evidence suggests that the subset of monocytes expressing CD16 (FcγRIII) may be important mediators of HIV-1 pathogenesis. These cells express low levels of CD14 and high levels of CD16. Such cells represent only 5-10% of the blood monocyte population in healthy persons (229) but can increase to levels of >40%, especially in HIV-1-infected patients with advanced disease and active opportunistic infection (230, 231). CD16+ monocytes are believed to be at a more advanced stage of differentiation compared to $CD16^-$ cells (223, 226) and they are more susceptible to HIV-1 infection than the classical $CD14^{high+}CD16^-$ subset of cells, presumably as a result of their increased CCR5 expression (228). They produce high levels of TNF-α (232) and it has been suggested that the increasing prevalence of these cells may, in part, be responsible for the progressive dysregulation of the cytokine network that occurs during disease progression. $CD14^{low+}CD16^{high+}$ monocytes are greatly expanded in patients with AIDS-related dementia (233) and it has been proposed that, during neuropathogenesis, increased numbers of these cells migrate into the CNS to become $CD14^{low+}CD16^{high+}CD^{163+}$ perivascular macrophages (234). Unlike microglia, these cells are continuously repopulated by blood-derived monocytes. Perivascular cells are uniquely situated at the

intersection between the nervous and immune systems and are believed to play an important role in a number of inflammatory diseases including bacterial meningitis and experimental allergic encephalomyelitis, as well as simian immunodeficiency virus (SIV) and HIV-1 encephalitis (234-238). Other studies have shown that upon differentiation into macrophages, and interaction with T cells, $CD16^+$ MDM-T cell conjugates become major sites of HIV-1 replication (239, 240).

As with monocytes, tissue macrophages are characterized by extensive heterogeneity and a high level of plasticity. They respond to, and are profoundly affected by, a wide range of immuno-modulatory cytokine, chemokine and microbial gene products that are present in different tissue environments (241-246). These interactions lead to the differential modulation of cytokines, chemokines and cell surface receptors, resulting in the emergence of distinct subsets of macrophages that differ in their functional and phenotypic properties. Alveolar macrophages, for example, express high levels of pattern recognition receptors and scavenger receptors that are involved in clearing viruses and other microorganisms, as well as environmental particles (247), whereas osteoclasts express markers that are fundamental to the remodeling of bone tissue (215, 248, 249). Macrophages located at sites of pathogen entry in the intestine have high phagocytic and anti-bacterial activity, but produce only low levels of inflammatory cytokines, a mechanism that prevents unnecessary over-stimulation due to constant exposure to commensal and pathogenic microbes (250-252). Tissue-specific differences can have a major effect on the capacity of macrophages to support productive HIV-1 infection.

Macrophages are widely distributed in peripheral tissues, including mucosal membranes. They play an important role in the establishment and dissemination of HIV-1 infection, and they serve as viral reservoirs throughout the course of HIV-1/AIDS (246). Alveolar, peritoneal and placental macrophages, as well as microglia are all susceptible to HIV-1, and depending on the local environment, the number of infected macrophages can range from 1% to 50% (253-255). Although macrophages serve as reservoirs of "latent"

infection, they can also support high levels of viral replication, especially during opportunistic infection (226, 255) and late-stage disease when CD4+ T cells have been severely depleted (226, 256). Macrophages are infected predominantly by CCR5-tropic viruses, however, viral isolates using CXCR4, CCR3 and CCR2b as the co-receptor can also infect and replicate in monocyte-derived macrophages (MDM) (257-260). Unlike CD4+ T cells, macrophages are relatively resistant to the cytopathic effects of HIV-1, and virus archived in long-lived macrophages such as microglia can survive for many years (218, 260, 261). The persistence of intracellular HIV-1 in sanctuary tissues such as the central nervous system (CNS), where the penetration of protease inhibitors (PIs) is restricted by the blood-brain, represents a major obstacle to the treatment-induced elimination of infection (226, 262, 263). Both protease and RT inhibitors are less effective in chronically infected macrophages compared to CD4+ T cells (226, 264, 265).

HIV-1 infection leads to the impairment of monocyte-macrophage function. This, in turn, results in inefficient control of opportunistic pathogens and further enhancement of immune activation and disease pathogenesis. Monocytes, alveolar macrophages and MDM obtained from HIV-1-infected patients have all been shown to exhibit reduced phagocytic capacity (266-268). This reduction is not due to the down-regulation of phagocytic receptors (complement, FcR), but instead appears to involve the inhibition of tyrosine phosphorylation and an impairment of downstream signaling pathways (260, 266). HIV-1 also inhibits phagosome-lysosome fusion and intra-cellular killing of opportunistic pathogens, and the degree of inhibition correlates with the stage of disease (260, 269). Monocytes obtained from patients with AIDS also have defective migratory responses due to a down-regulation of receptors for chemotactic ligands (ie. C5a and fMLP) (270, 271). Chronic immune activation of monocytes and macrophages by HIV-1 or other antigenic stimuli, leads to altered secretion of pro- and anti-inflammatory cytokines and chemokines, and ultimately to dysregulation of the host immune system.

1.4 ROLE OF GASTROINTESTINAL TRACT IN HIV-1 PATHOGENESIS

1.4.1 Overview of the Intestinal Immune System

The gastrointestinal mucosa is located at the interface between a sterile internal and a microbially contaminated external environment. It serves the conflicting needs of nutrient absorption and host defense, functions that require intimate contact with the external environment (272, 273). As a result, the GIT has evolved a thin polarized epithelium and a large surface area characterized by mucosal folds, villi and microvilli. In the small intestine of healthy persons, antigen exposure comes primarily from the diet, whereas in the ileum and colon the antigenic load is further enhanced by an abundant and complex array of commensal microbes (274). The organization of the epithelial barrier with its tight junctions helps reduce the risk of luminal antigens entering the mucosa (including commensal and pathogenic microbes), but it does not completely prevent this process (274-276). Some food proteins and non-pathogenic commensal bacteria enter the mucosa through breaks in the tight junctions, possibly at the villus tip where epithelial cells are shed, a process that is relatively limited in healthy individuals (277). The task of mounting an effective immune response to invading pathogens while remaining relatively unresponsive to commensal microbes and food antigens poses a daunting challenge to the GIT.

As a result, the gut mucosa has evolved an elaborate system to protect its host from infectious agents. This system consists of two anatomically separated but functionally linked components of the common mucosal immune system (273). An afferent component, which contains elements involved in the initiation of the immune response including antigen presentation and lymphocyte proliferation, and an efferent component containing elements directly involved in antibody production and CTL responses. Structurally, the afferent system consists of distinct lymphoid follicles overlaid by an epithelial membrane containing M cells. These cells transcytose particulate antigens to antigen-presenting macrophages located at the basal surface of the epithelium (278, 279). Macrophages are

the first phagocytic cells to interact with microorganisms that have entered the intestinal mucosa. Intestinal macrophages have avid phagocytic and bactericidal activities that protect the host from pathogenic organisms and they regulate the immune response to commensal bacteria (discussed in more detail below) (251). Antigen-presenting DC survey the microenvironment by extending processes between gut epithelial cells. They sample both commensal and pathogenic microbes for subsequent transport and presentation to B and T lymphocytes in the spleen and lymph nodes (280, 281). Cells of the efferent compartment are diffusely scattered throughout the epithelium and lamina propria of the intestine. In addition to CTL, IFN-γ-producing lymphoctyes and IgG/IgA-secreting plasma cells, antibody-dependent cytotoxicity may also play an important role in the adaptive immune response (273, 282-284). Together, this defense system consisting of the innate immune system, the epithelial barrier and its associated mucous layer and the adaptive immune system, can effectively prevent or restrict the entry and propagation of commensal and pathogenic organisms, including HIV-1 (252).

Recently, in North America, there has been a dramatic increase in inflammatory bowel disease in the absence of overt microbial infection (274). This finding suggests that some, as yet unknown factor, is perturbing the balance between the normal microflora of the gut and host immunity. It will be important to determine whether this change is due to the use of antibiotics and a treatment-associated reduction in commensal flora. It has been suggested that, under normal homeostatic conditions, the anti-inflammatory responses induced by commensal flora protect the intestinal epithelium from pathogenic insults (285, 286). This relationship, however, appears to extremely delicate and anything that perturbs either immune or epithelial homeostasis can lead to inflammation and life-long inflammatory conditions such as Crohn's disease and ulcerative colitis. Patients afflicted by these disease suffer from chronic diarrhea, weight loss and fatigue, in addition to other potential complications such as skin ulcers, arthritis and bile-duct inflammation (287).

1.4.2 Intestinal Macrophages as Unique Effector Cells

The mucosa of the GIT contains the largest reservoir of macrophages in the body (288). These cells, which form part of the innate immune system, are the first line of defense against microorganisms that have entered the mucosa. Macrophages in the intestinal mucosa have developed a unique and highly distinctive set of phenotypic and functional properties that are not found in other tissue macrophages, properties that promote the phagocytosis and elimination of harmful pathogens while, at the same time, protecting the gastrointestinal mucosa from chronic over-stimulation and inflammation (**Figure 1.8**).

Accumulated evidence suggests that intestinal macrophages are continually replenished by blood monocytes that are recruited in response to endogenous or inflammatory chemokines. Following exposure to transforming growth factor-β (TGF-β) and other stromal factors present in the lamina propria, these monocytes mature into resident macrophages. Unlike lymphocytes, these tissue macrophages do not proliferate and they lack chemotatic activity (217, 252). Resident intestinal macrophages express high levels of HLA-DR and aminopepetidase N (CD13), but exhibit a profound down-regulation of innate response receptors, including receptors for bacterial lipopolysaccharide (LPS) (CD14), for IgA/IgG (CD89, CD16, CD32 and CD64), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) (252). Growth factor receptors involved in the production of pro-inflammatory cytokines including IL-2 (CD25) and IL-3 (CD123) and the integrin LFA-1 (CD11a/CD18) are also down-regulated in resident macrophages (252). As a result, intestinal macrophages in non-inflamed tissues do not produce pro-inflammatory cytokines such as IL-1, IL-6, IL-10, IL-12, CCL5/RANTES, TGF-β and TNF-α (251, 252, 289). They also do not express CCR5 and CXCR4 (290, 291), the primary co-receptors for HIV-1, or CD80, CD86 and CD40, co-stimulatory molecules required for the induction of T cell activation (292). To facilitate pathogen identification, however, intestinal macrophages express a number of pattern recognition receptors including the toll-like receptors (TLR)

such as TLR5 and TLR9 which are capable of interacting with foreign carbohydrate and lipid structures to promote the clearance of harmful pathogens in the absence of inflammation (252, 289).

In contrast to the profound down-regulation of pro-inflammatory markers that is observed in the non-inflamed intestine, monocytes recruited to an inflamed intestinal mucosa retain their potential to develop into pro-inflammatory cells. For example, up to 30% of the intestinal macrophages in inflammatory bowel disease (IBD) patients express CD14 and have the potential to secrete pro-inflammatory cytokines (293, 294). They also express the triggering receptor expressed on myeloid cells- (TREM-1), a finding that may explain the respiratory burst activities frequently observed in IBD patients (295, 296). Intestinal macrophages in the inflamed mucosa also exhibit increased co-stimulatory activities as measured by the up-regulation of CD80, CD86 and CD40 (297, 298).

1.4.3 HIV-1 Enteropathy, Microbial Translocation and Immune Activation

HIV-1-associated enteropathy is a poorly-defined clinical condition in which chronic diarrhea, malabsorption and wasting occurs in the absence of a detectable enteric pathogen (273, 299). Whether this condition is due to subclinical enteric infections, chronic inflammation or direct effects of HIV-1 on the epithelium of the GIT remain to be established. Although there is little evidence that HIV-1 infects enterocytes, various histological studies have reported that the intestinal mucosa of HIV-1-infected persons is characterized by chronic inflammation, villous atrophy, crypt hyperplasia, nuclear enlargement and apoptosis. It has also been reported that jejunal enterocytes have an over-developed smooth endoplasmic reticulum and decreased levels of mitochondria (300-302). *In vitro* studies, performed on duodenal biopsies have shown that HIV-1-infected patients with diarrhea have decreased trans-epithelial resistance when compared to patients without diarrhea (303). At the molecular level, the binding of gp120 to GPR15/Bob expressed on

the basal surface of epithelial cells has been shown to cause decreased acetylation of tubulin, microtubular depolymerization and cytoskeletal rearrangements, changes that result in increased intestinal permeability, increased levels of intracellular calcium and diarrhea (304). Other studies have reported that HIV-1 infection is associated with degenerative changes in the enteric nerves and in the vasculature of the lamina propria (305, 306) and that the protease inhibitors, saquinavir, ritonavir and nelfinavir (but not indinavir) cause damage the epithelial barrier (307). Irrespective of the mechanism(s), HIV-1 enteropathy appears to be a subtle phenomenon.

Studies showing massive depletion of CD4+ T cells in the GALT during the first few weeks of infection have suggested that this loss in helper T cell function together with the damage caused to the epithelium, may alter the antimicrobial properties of the gut allowing for the increased translocation of luminal microbes and microbial products (112, 121). This phenomenon, known as microbial translocation, can occur in the absence of overt viremia and is a prominent feature of IBD (308). Damage to the intestine during invasive intestinal surgery and during immunosuppressive conditioning for bone marrow transplantation can also increase the level of microbial translocation (308, 309). This leads to both local and systemic immune activation and, in the case of transplant patients, an intensification of graft-versus-host disease (GVHD) (309). Quantification of plasma LPS, a major component of the cell wall of Gram-negative bacteria (310) is routinely used to measure the extent of epithelial leakage and bacterial translocation (311, 312).

Based on preliminary data showing that microbial translocation may be a cause of systemic immune activation in chronic HIV-1 infection, a new model of HIV-1 pathogenesis is beginning to emerge. In this model, the level of immune competency that exists after the acute phase of infection, especially as it relates to the control microbial pathogens in the GIT, determines the rate of disease progression to AIDS (112). It has been known for some time that markers of immune activation are better predictors of HIV-1 disease progression than CD4+ T cell counts and plasma viral load (313). However, the

factors driving immune activation, including polyclonal B cell activation, increased activation and turnover of T cells and increased levels of circulating pro-inflammatory cytokines and chemokines remain unclear (314-317). Evidence supporting the view that microbial translocation is an important cause of systemic activation is based on studies showing that: 1) LPS is significantly increased in the plasma of patients with chronic HIV-1 infection; 2) plasma LPS correlates with other measures of innate and adaptive immune activation; 3) long-term non-progressors have undetectable to low-level LPS in association with low levels of immune activation; 4) antiretroviral therapy leads to a partial reduction in circulating LPS, and 5) non-pathogenic SIV infection of sooty mangabeys does not lead to increased levels of LPS or immune activation, despite extensive CD4+ depletion in the GIT (112, 121).

1.5 ANTIRETROVIRAL THERAPY (ART)

1.5.1 Historical Perspective and Current Drug Regimens

Following the identification of HIV-1 as the causative agent of AIDS, many scientists focused their efforts on developing a therapeutic strategy aimed at eliminating the virus. The very first antiretroviral drug was zidovudine (ZDV), a nucleoside analog that competitively inhibits the viral reverse transcriptase (RT) by inducing chain termination and an interruption of HIV-1 cDNA synthesis (318). Unfortunately, shortly after its discovery and approval by the FDA, scientists discovered the limitations of monotherapy (319, 320). It became increasingly clear that, because of the ability of HIV-1 to rapidly generate drug-resistant mutations, a combination of drugs would be required to slow the emergence of resistance and induce a more profound and sustained suppression of viral replication. With an increased understanding of the HIV-1 life cycle, additional viral proteins, most notably the protease enzyme were identified as potential therapeutic targets. This led to the development of the PIs, a highly potent class of therapeutic agents that bind directly to the active site of the HIV-1 protease enzyme, preventing virion maturation and

the formation of infectious virus (321, 322). Soon thereafter, triple combination therapy consisting of a PI and two nucleoside RT inhibitors (NRTIs), ultimately referred to as highly active anti-retroviral therapy (HAART), was introduced as the standard of care for HIV-1+ patients in the developed world (323). Subsequently, a third class of anti-HIV-1 agents, the non-nucleoside RT inhibitors (NNRTIs) became available. NNRTIs are a structurally diverse group of drugs that bind to the HIV-1 RT at a position distal from the active site, causing conformational changes in the reactivity of the active site (321). In common with PIs, NNRTIs have a synergistic effect when used in combination with two different NRTIs (321).

Other treatment options that are in an advanced stage of evaluation include two promising entry inhibitors, Enfuvirtide and Maraviroc. Enfuvirtide, or T20, is a fusion inhibitor that has been approved for use in treatment-experienced patients (324-326). It is a synthetic peptide that mimics amino acids 127-162 of HIV-1 gp41, a key domain involved in viral fusion with the cell membrane. Maraviroc, on the other hand, binds with CCR5 preventing interaction with HIV and CCR5-mediated signaling events (327). Maraviroc is currently under investigation in expanded phase III clinical trials (327, 328).

1.5.2 Emerging Drug Targets and Future Directions

Concerns about toxicity and the emergence of drug resistance continue to drive the search for simple effective low-cost drugs that can be used to boost the therapeutic efficacy of current regimens. Such drugs are needed, not only to reduce the risk of toxicity and resistance, but also to provide treatment options for patients who have failed their first- and (or) second-line drug regimens. In addition to the entry inhibitors described above, drugs that target the viral integrase are showing significant therapeutic promise, primarily because of the unique enzymatic activity of the viral IN. Two potential approaches are possible: one that uses small molecular inhibitors to directly target the active site of integrase and a second approach that blocks formation of the Pre-Integration Complex

(PIC) (329). Most integrase inhibitors currently under development block the transfer activity of the enzyme. Unfortunately, like all anti-HIV-1 compounds, resistance mutations develop during extended exposure to integrase inhibitors (330).

HIV-1 assembly and budding also remains a significant unexploited target for antiretroviral therapy. While anti-budding agents, so far, have not been practical due to their significant cellular toxicity, inhibitors targeting TSG101-p6 interactions may prove valuable (329). In addition, Vpu plays an important role in virus budding from human cells and may be a potential target for antiretroviral therapy. Specifically, recent studies have shown that Vpu restricts the antiviral activity of tetherin, an integral membrane protein that binds to and retains fully formed virions on the surface of infected cells (331). Tetherin is thought to represent a late stage antiviral barrier preventing the dissemination of HIV-1 thus targeting Vpu-tetherin interactions may represent a novel therapeutic target (331).

Finally, it has been suggested that innate antiviral mechanisms may provide an important new class of therapeutic agents. These host targets are particularly attractive since they do not mutate in response to drug selection pressure and therefore do not develop resistance. APOBEC3G is a particularly attractive candidate. APOBEC3G is a DNA editing enzyme that exerts its antiviral activity by introducing C-to-U changes in the newly synthesized viral single stranded cDNA (332). However, in the setting of HIV-1, the antiviral activity of APOBEC3G is negated by the presence of HIV-1 Vif, a protein that is incorporated into HIV-1 virions and targets APOBEC3G for proteosomal degradation (332). Small molecular inhibitors directed against the Vif-APOBEC3G interaction may provide a novel therapeutic option.

1.5.3 Success of ART in the North America and Europe

Since the introduction of ART 28 years ago, substantial progress has been made in both the treatment and prevention of HIV-1/AIDS, especially in resource-rich regions of the developed world where drugs are readily available. In the USA alone, it is estimated

that more than 3 million years of life have been saved since 1989, as a direct result of progress in AIDS care and treatment, including prophylactic treatment for the prevention of opportunistic infections (OIs) and treatment with antiretroviral therapy (ART) (333). In addition, prevention of mother-to-child transmission efforts averted an estimated 2,900 childhood infections, the equivalent to an additional 137,000 years of survival benefit (333, 334). Cost-effective analyses have suggested that, overall, prevention efforts in the USA have averted between 204,000 and 1,585,000 infections at a cost that is less than that required to treat an individual patient for HIV-1-related disease (335). Similar levels of success have been observed in other resource-rich regions of the world, including Western Europe and Australia. Despite these advances, significant challenges remain if HAART is to be universally implemented (see Section 1.5.5). Many of the challenges, in the developing world, relate the limited repertoire and high cost of drugs, the lack of clinical and laboratory infrastructure, the shortage of health care workers and the high prevalence of opportunistic co-infections, as well as universal concerns relating to drug toxicity and the emergence and transmission of drug resistance.

1.5.4 Immune Reconstitution Inflammatory Syndrome (IRIS)

Combination antiretroviral therapy has led to significant declines in AIDS-associated morbidity and mortality, reducing the frequency of opportunistic infections and prolonging survival (336-338). These benefits appear to be the result of a partial recovery in the host's immune system, as manifested by an increase in CD4+ T cells and a decrease in plasma viral loads (339). In some patients, treatment-induced immunological change, which includes a rapid restoration of pathogen-specific immunity, is associated with clinical deterioration despite increased levels of CD4+ T cells and decreased HIV-1 loads (340-342). This condition, known as the "Immune Reconstitution Inflammatory Syndrome" (IRIS) usually occurs within the first 3 months after the initiation of therapy (343). IRIS is often self-limiting but can, in some patients, lead to significant morbidity and death.

Accumulated evidence suggests that IRIS represents an inflammatory response or a "dysregulation" of the immune system to an antigenic stimulus. This stimulus can be a viable infectious agent, a dead or dying infectious organism, or a tumor antigen such as HHV-8, the causative agent of Kaposi's sarcoma (KS) (344-346). Two different types of IRIS have been described - an "unmasking" form which is directed against a previously untreated subclinical (replicating) pathogens and a "paradoxical deterioration" form directed against residual antigens of dead or dying organisms that have been previously diagnosed and are being treated (343). The most common forms of IRIS are due to mycobacterial infections (tuberculosis, non-tuberculous mycobacteria, leprosy), fungi (*cryptococcus, pneumocystis, histoplasma, candida*), herpes simplex virus (HSV), herpes zoster virus (HZV), and cytomegalovirus (CMV), although protozoa (*toxoplasma, microsporidia, Lesihmania, cryptosporidia*), helminth (*schistosoma*) and non-herpes viruses (hepatitis B (HBV) and hepatitis C (HCV)) can also induce IRIS (343).

IRIS appears to be more common in patients with low CD4+ counts (<200 cells/mL) (340, 347) and in patients who exhibit an increased rate of CD4+ T cell recovery during ART (348, 349). In most cases, CD4+ T cell recovery occurs within the first 1-2 weeks after starting treatment and continues for 2-3 months. The initial recovery is due primarily to a rapid redistribution of activated CD4+CD45RO+ memory T cells from lymphoid tissues (350, 351). These cells are primed to recognize previous antigenic stimuli and thus, may contribute to the early manifestations of IRIS. This is followed by an increase of naïve T cells, a process that is responsible for the quantitative increase in CD4+ T cell counts (352). Thus, the clinical manifestations of IRIS may be due to a combination of quantitative restoration of immunity and qualitative changes in T cell function and phenotype. Alteration in the Th1/Th2 cytokine balance may also help explain some of the manifestations of IRIS (353). Increased levels of IL-6, CCR3 and CCR5 have been detected on monocytes and granulocytes of patients who have a history of IRIS (354). Factors limiting our understanding of IRIS relate to the small sample size of most studies,

the retrospective nature of the studies and the fact that most analyses are based on peripheral blood rather than tissue specimens collected from lymphoid organs, or from the site of infectious disease (343).

1.5.5 Constraints to ART in Africa and Resource-Poor Settings

Treating and caring for millions of Africans living with HIV/AIDS poses an inescapable challenge to the continent and the world in general. According to UNAIDS, as of December 2007, an estimated 3 million people in low- and middle-income countries were receiving antiretroviral drugs (1). This represents 31% of those who need medication. However, little is known about the long-term efficacy of HAART in African populations infected with non-B subtypes. In a recent U.K. study, African patients had good initial responses to HAART but the duration of the response was shorter than that observed for Caucasians with a number of patients showing viral rebound after nine months (355). Although the reasons for the more limited response in African patients are not known, they are likely to be complex. One potential reason may be related to the lack of diagnostic and laboratory monitoring (viral load, CD4+ T cell counts) and financial resources. As a result, treatment is often initiated too late in the course of infection to prevent high mortality rates. Patients started on late therapy have a high incidence of concurrent viral and bacterial infections, such as tuberculosis (356). These infections cause chronic activation and dysregulation of the host immune system making it difficult to control viral replication and dissemination. Such patients may also be more likely to suffer from IRIS. Finally, as in the developed world, toxicity and resistance is always a concern.

In addition to being simple, safe and affordable, effective anti-retroviral treatments for Africa need to be pregnancy friendly and compatible with TB treatment (given the high incidence of TB/HIV-1 and TB/KS/HIV-1 and other multiple infections) (357). As stated previously, the successful treatment of HIV-1/AIDS in Africa will depend on the development of therapeutic strategies for treating OIs, either prior to, or concurrently with

HAART. At the present time, regimens that combine two NRTIs, zidovudine (ZDV) or stavudine (d4T) and lamivudine (3TC), with one NNRTI, either nevirapine (NVP) or efavirenz (EFV), are recommended as first-line therapy in South Africa (358).

1.6 RATIONALE AND AIMS OF THE CURRENT STUDY

1.6.1 Relevance to Existing Knowledge

Three decades into the global AIDS pandemic our understanding of HIV-1 pathogenesis continues to evolve. It has become increasingly apparent that generalized immune activation is a key pathogenic determinant in the development of AIDS and that immune activation is a better correlate of disease progression than either CD4 counts or plasma viral load (112, 313). Many new insights have emerged from comparative studies of natural vs. pathogenic SIV infection. Sooty mangabeys, the natural hosts of SIV, do not progress to AIDS despite high viral loads and mucosal CD4+ T cell depletion (179). In contrast, SIV infection of rhesus macaques has a disease course similar to HIV-1 in human. The fundamental difference driving pathogenesis appears to be sustained immune activation following the resolution of acute infection (179). Based on this information, the important questions then become: What are the mechanisms driving chronic immune action in HIV-1-infected persons? Is this immune activation preventable? Can an improved understanding of immune activation be used to design improved prevention and treatment strategies?

Studies of immune activation, in the setting of HIV-1, have focused primarily on components of the cellular immune response. However, recent data suggests that microbial translocation and high levels of circulating LPS may be the cause of systemic immune activation in HIV-1-infected patients and, by inference, that the innate immune system may play an important role in driving HIV-1-associated pathogenesis, either directly or indirectly (121). Monocytes and macrophages, the primary targets of LPS-induced

activation, are key regulators of inflammation. They are responsive to a wide range of microbial and environment stimuli and depending on the type of stimuli can undergo functional polarization to either an "immuno-responsive/inflammatory" or a "tissue remodeling" phenotype (359). Cells of monocyte-macrophage lineage are also susceptible to HIV-1 infection and can serve as long-lived viral reservoirs during ART (226, 260). HIV-1 infection of monocytes and macrophages also leads to impaired phagocytosis, chemotaxis and cytokine production (260).

1.6.2 Aims of the Current Study

Given the importance of immune activation in driving progression to AIDS, the overall aim of the current study was to obtain a comprehensive understanding of the relationships between MPS activation/polarization and HIV-1 pathogenesis. A main focus of the study was to investigate the *in vitro* and *ex vivo* potential of these cells to serve as targets of HIV-1 infection and mediators of immune and inflammatory responses.

Specific aims, at the *in vitro* level, were to determine how cytokine-induced polarization of monocyte-derived-macrophages (MDM) can alter the phenotype and functional properties of MDM and affect their ability to support productive vs. latent infection. An additional aim was to examine the affects of polarization on the ability of MDM to transmit HIV-1 to susceptible $CD4^+$ T cell targets. These studies were designed to obtain new insights into the relationship between macrophage polarization and the capacity of these cells to serve, not only as targets and reservoirs of infection, but also as vehicles of viral dissemination and spread to T cells. This work also serves as a baseline for studies aimed at elucidating the activation profiles of blood monocytes and tissue macrophages at the clinical level.

Given that immune activation may play a particularly important role in driving HIV-1 pathogenesis in Africa and other parts of the developing world, clinical studies were conducted in treatment naïve South African patients infected with subtype C, a viral

variant that constitutes >50% of global infections. The overall aim of this work was to obtain new insights for the immune-based enhancement of antiretroviral therapy in patients with late stage disease. More specific aims included establishing an immune activation profile for blood monocytes and identifying factors driving the activation of these cells (ie. microbial translocation vs. HIV-1-induced activation). The final aim of the study, given the importance of mucosal tissues in HIV-1 pathogenesis, was to investigate the affects of HIV-1 infection on intestinal macrophagess in the duodenum and colon. Since intestinal macrophages drive chronic inflammation and immune dysfunction in IBD, it seemed reasonable to investigate whether these cells play a similar role during HIV-1 infection.

In summary, the main overall objective of the study was to examine the relative contribution of MPS activation/polarization to the chronic immune activation and dysfunction associated HIV-1 disease. MP may be functionally important in driving this phenomenon since they play a pivotal role at the interface between immune activation, HIV-1 pathogenesis and microbial translocation. An increased understanding of these different parameters disease pathogenesis may lead to the design of novel treatment strategies aimed at reducing systemic immune activation.

1.7 FIGURE LEGENDS

FIGURE 1.1. Global view of HIV-1 infection in 2007. Map showing the distribution and frequency of HIV-1/AIDS in different regions of the globe relative to the total number of HIV-1 infections worldwide as presented in the UNAIDS report on AIDS for 2008.

FIGURE 1.2. HIV-1 subtypes and CRFs. Map showing the unequal distribution of HIV-1 subtypes and Circulating Recombinant Forms (CRFs) at the global level. These maps are based on prevalence data contained in the UNAIDS reports on AIDS and on sequence data available through the Los Alamos National Laboratory (LANL) database (from Taylor et al. N Engl J Med 2008; 358:1590).

FIGURE 1.3. Overview of the HIV-1 life cycle. The infectious life cycle begins when the HIV-1 gp120 glycoprotein binds to the CD4 receptor on the surface of helper T cells, macrophages or dendritic cells (Step 1.). This interaction induces conformational changes in the core region of gp120, leading to unmasking of the chemokine co-receptor (CCR5 or CXCR4) binding site. Further interaction with CCR5 (or CXCR4) leads to further conformational changes, activation of gp41 and virus fusion with the cell membrane (Step 2.). The viral core is then extruded into the cytoplasm where it is reverse-transcribed into cDNA before being transported to the nucleus and integrated into the human genome, a process that is catalyzed by the HIV-1 integrase enzyme (Step 3.). Transcription of the integrated proviral DNA leads to the production of multiply, singly and unspliced mRNAs that are then transported back to the cytoplasm where they are translated and processed into HIV-1 structural and accessory proteins (Step 4.). Env and immature precursors of the capsid (Gag) and viral polymerase (Pol) are transported to the cell membrane and assembled into HIV-1 virions. Virus infectivity is acquired after Gag and Gag-Pol cleavage, particle maturation and budding from the cell surface (Step 5.). Most currently available drugs target the reverse transcription (nucleoside and non-nucleoside RT

inhibitors) or the protease cleavage step required for the development of infectivity (from Monini et al. Nat Rev Cancer 2004;4:861).

FIGURE 1.4. Schematic showing HIV-1-associated damage to the gastrointestinal tract (GIT). (a) A healthy GIT consisting of an intact epithelial barrier with villi and crypts, a lumen containing secreted IgA and defensins and a lamina propria containing an abundance immune cells including macrophages, dendritic cells, B cells and T cells. (b) An HIV-1-infected GIT showing damage to the epithelial barrier, enterocyte apoptosis, high-level viral replication, massive CD4+ T cells depletion and increased membrane permeability in association with increased microbial translocation (from Mowat et al. Nat Rev Immunol 2003;3:331).

FIGURE 1.5. Characteristics of T cell Exhaustion during chronic viral infections. Virus-specific $CD8^+$ T cells responses are complex, ranging from cytokine (IFN-γ, TNF-α and IL-2) production to protection from apoptosis and antigen-induced T cell proliferation. Many of these functions, including cytokine production and the proliferative potential of T cells, are progressively exhausted during the course of chronic infection. Factors contributing to this exhaustion are chronic antigen-driven immune activation and the severe depletion of $CD4^+$ helper T cell function. Increased expression of the Programmed Death receptor, PD-1 by virus-specific T cells, and its corresponding ligand, PD-L1 on antigen presenting cells are also major contributors to T cell exhaustion during the chronic phase of viral infections, including HIV-1 (from Freeman et al. J Exp Med 2006; 203:2223)

FIGURE 1.6 Schematic showing the similarities and differences between pathogenic (human, rhesus macaques) and non-pathogenic retroviral infections. Although rapid and severe depletion of mucosal $CD4^+$ T cells during the acute phase of HIV-1 (and SIV) infection is an important determinant of pathogenesis, it is not sufficient to cause AIDS/SAIDS. Continued immune activation and enhanced microbial translocation during

the chronic phase of HIV-1/SIV infection are now believed to the primary factors driving disease progression and the ultimate collapse of the immune system (Paiardini et al. AIDS Rev 2008;10:36-46).

FIGURE 1.7 Schematic demonstrating how HIV-1 exploits DC-SIGN to circumvent antigen processing by dendritic cells and efficiently transfer virus to $CD4^+$ T cells. (a). HIV-1 is captured by DC-SIGN+ DC, internalized into lysosome-like vesicles and transported to distal lymphatic tissues protected from neutralizing antibodies, cytotoxic T cell responses and other host defense mechanism. Upon arrival at lymphoid tissues, the virus recycles back to the DC surface and is trans-transmitted to $CD4^+$ T cells. (b) In the presence of high levels of cell-free virus, DC-SIGN may enhance virus presentation to the $CD4^+$/co-receptor complex, promoting virus entry and productive infection of DC. The newly synthesized virions are released into the circulation for subsequent infection of new $CD4^+$ target cells. (c) DC-SIGN may also function as antigen presenting receptor to internalize and process viral antigens for presentation to T cells via MHC class I and II molecules (van Kooyk et al. Nat Rev in Immunol 2003;3:697).

FIGURE 1.8 Summary of the phenotypic and functional differences between intestinal macrophages and blood monocytes. In the healthy mucosa, monocytes recruited to the lamina propria acquire a distinct "anergic" phenotype as they become mature into resident macrophages. Although these cells remain HLA-DR, CD13, CD36 and TGF-β positive and retain their potent phagocytic and bactericidal properties, they do not express innate response or co-stimulatory receptors, including receptors for lipopolysaccharide (LPS), IgA, CR3, CR4, CD40, CD80, CD86, TREM-1 or the HIV-1 co-receptors, CCR5 and CXCR4 (from Smith et al. Immunol Rev 2005;206:149).

1.8 FIGURES

Figure 1.1

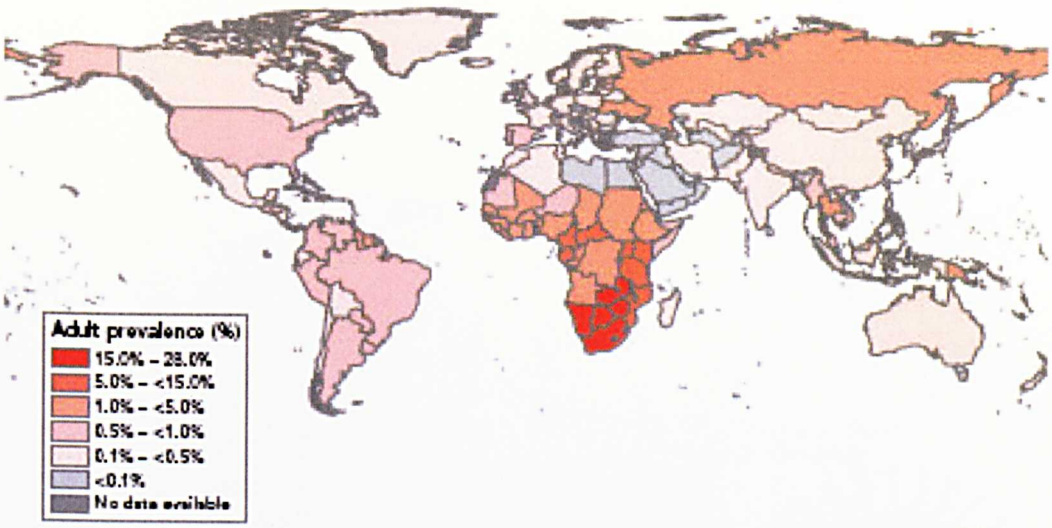


Figure 1.2

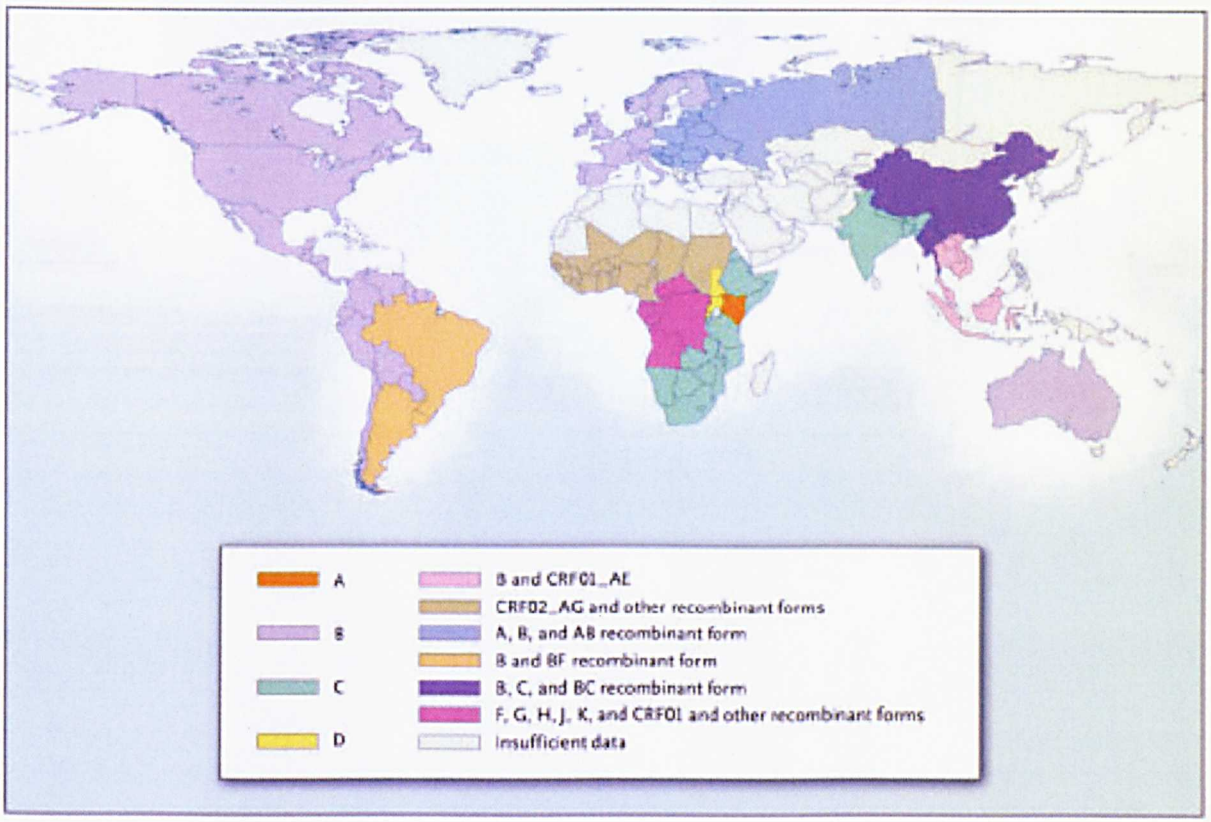
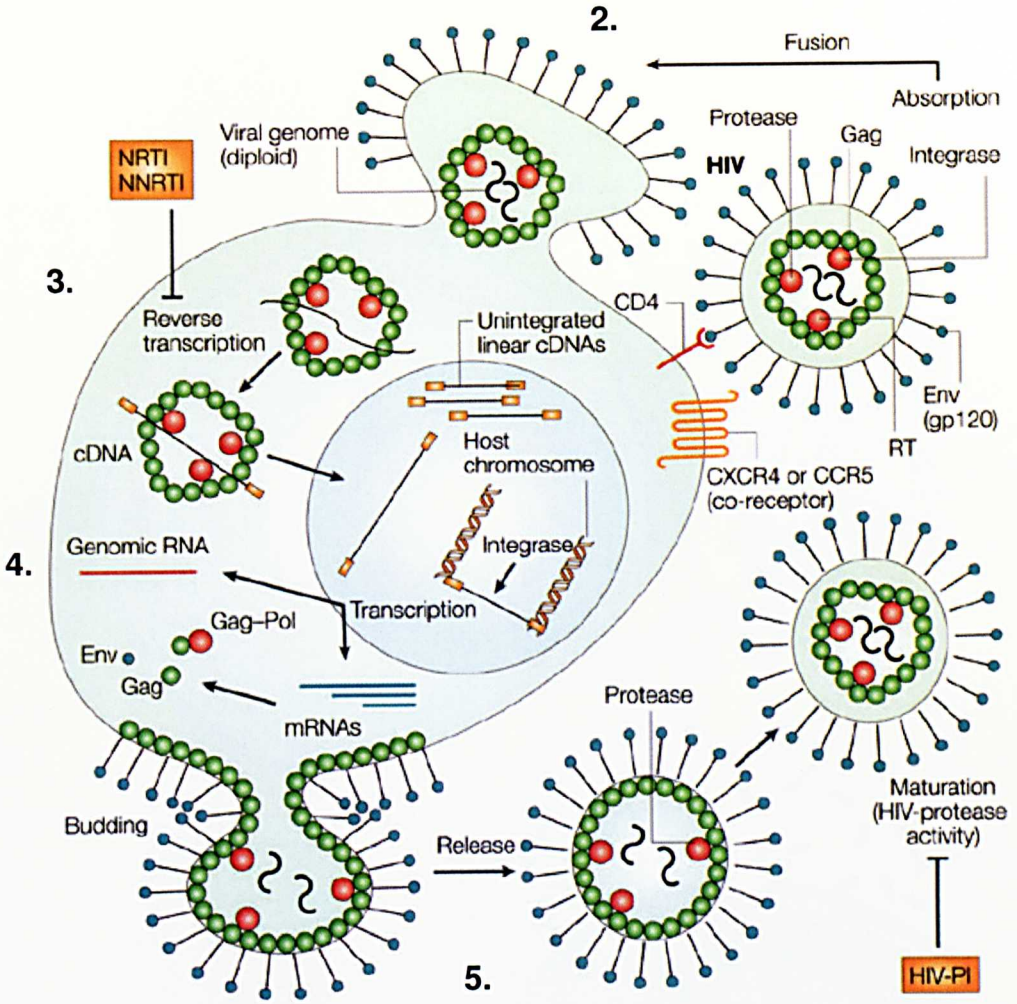


Figure 1.3


2.
Fusion
Absorption
Protease
Gag
Integrase
1.
HIV
Viral genome
(diploid)
NRTI
NNRTI
3.
Reverse
transcription
Unintegrated
linear cDNAs
CD4
Env
(gp120)
RT
cDNA
Host
chromosome
CXCR4 or CCR5
(co-receptor)
4.
Genomic RNA
Integrase
Transcription
Gag–Pol
Env
Gag
mRNAs
Protease
Maturation
(HIV-protease
activity)
Budding
Release
5.
HIV-PI
Nature Reviews | Cancer

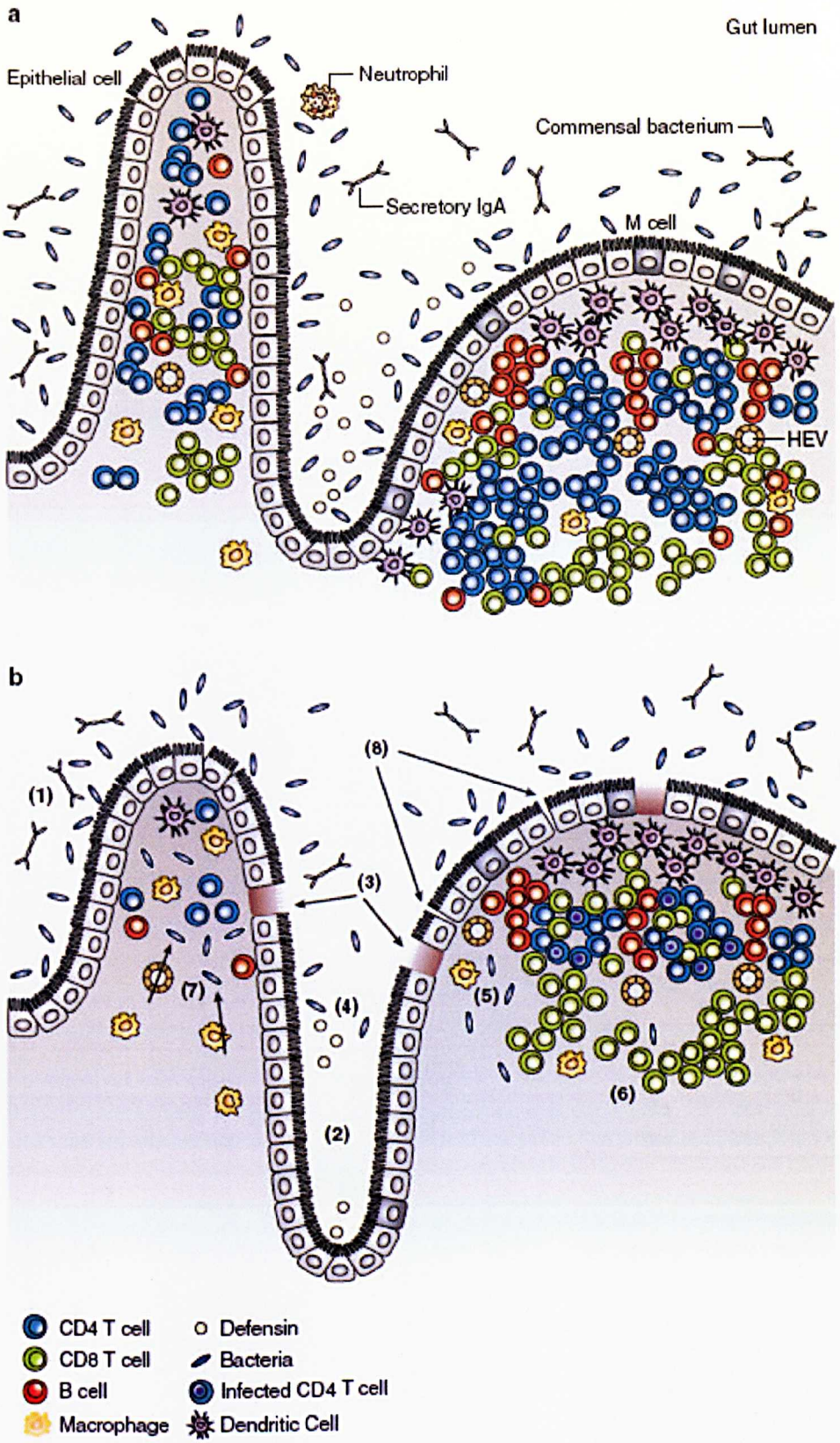

a
Gut lumen
Epithelial cell
Neutrophil
Commensal bacterium
Secretory IgA
M cell
HEV
b
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
CD4 T cell
CD8 T cell
B cell
Macrophage
Defensin
Bacteria
Infected CD4 T cell
Dendritic Cell

Figure 1.5

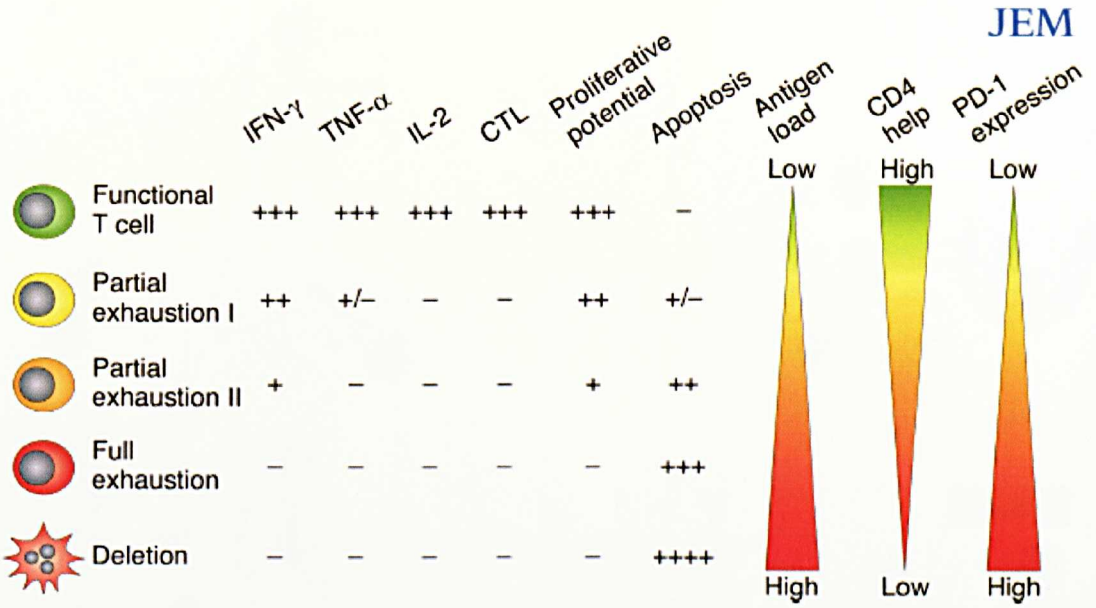
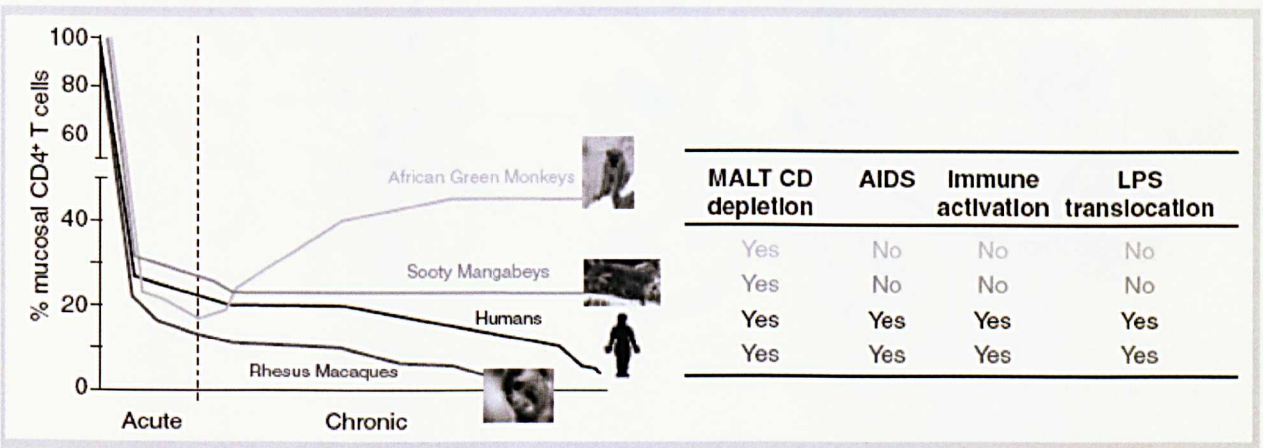
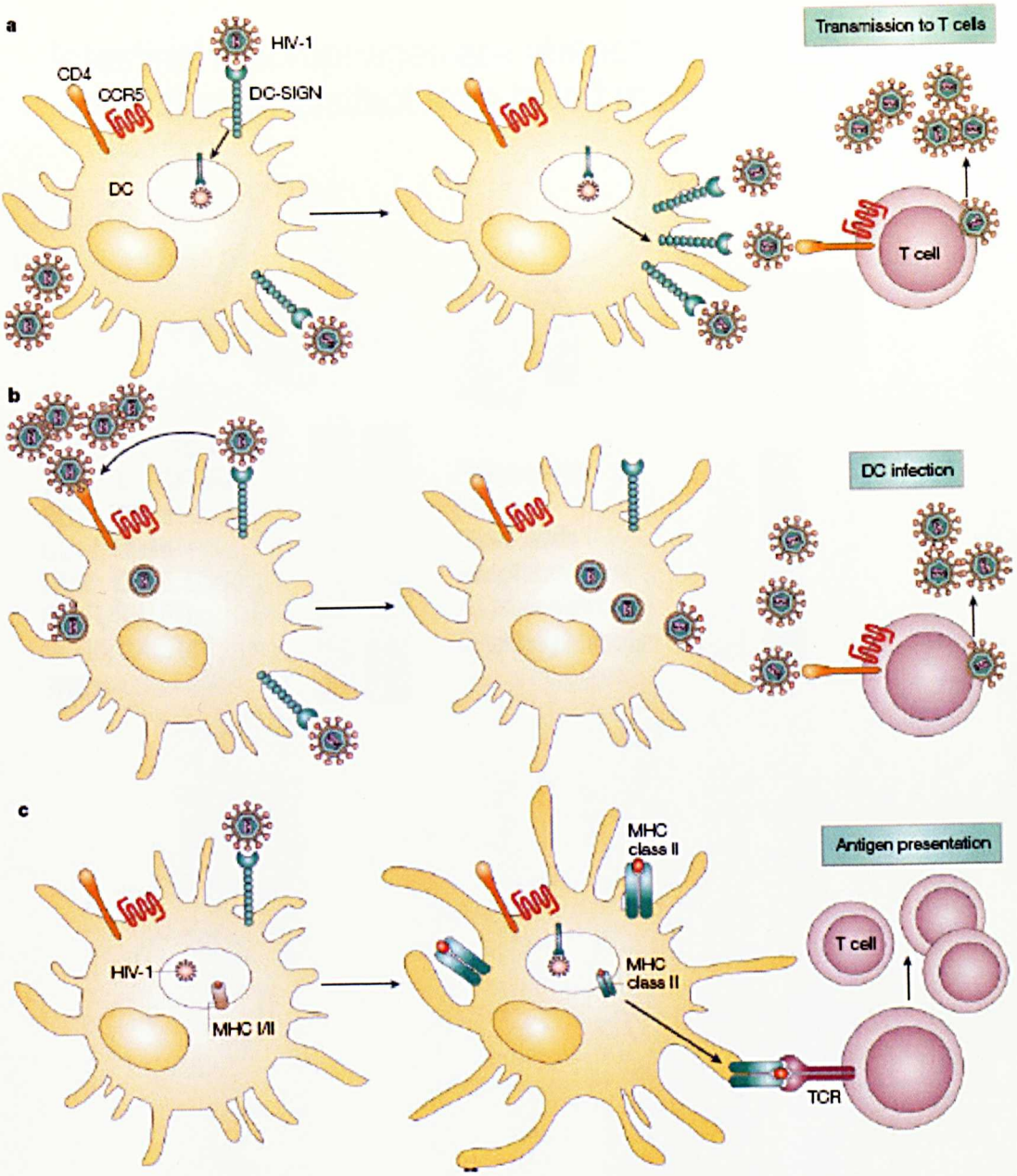


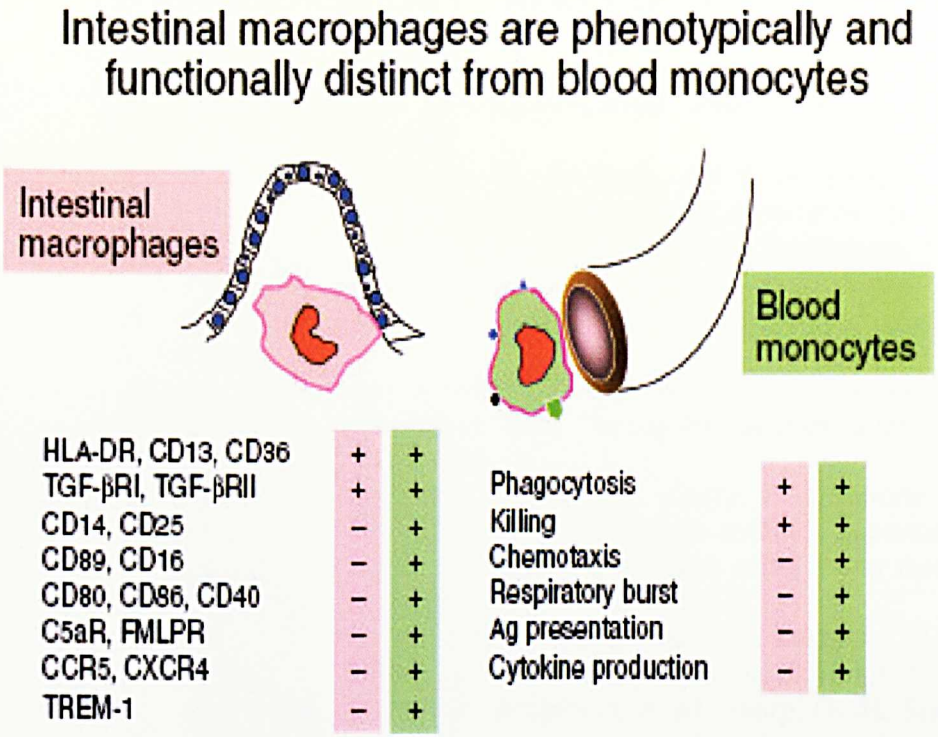
Figure 1.6



MALT CD depletion	AIDS	Immune activation	LPS translocation
Yes	No	No	No
Yes	No	No	No
Yes	Yes	Yes	Yes
Yes	Yes	Yes	Yes

Figure 1.7


a
HIV-1
CD4
CCR5
DC-SIGN
DC
Transmission to T cells
T cell
b
DC infection
c
HIV-1
MHC I/II
MHC class II
MHC class II
Antigen presentation
T cell
TCR


Intestinal macrophages are phenotypically and functionally distinct from blood monocytes
Intestinal macrophages
Blood monocytes
HLA-DR, CD13, CD36 + +
TGF-βRI, TGF-βRII + +
CD14, CD25 – +
CD89, CD16 – +
CD80, CD86, CD40 – +
C5aR, FMLPR – +
CCR5, CXCR4 – +
TREM-1 – +
Phagocytosis + +
Killing + +
Chemotaxis – +
Respiratory burst – +
Ag presentation – +
Cytokine production – +

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CHAPTER 2

PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF CLASSICALLY (M1) AND ALTERNATIVELY (M2a) ACTIVATED HUMAN MONOCYTE-DERIVED MACROPHAGES.

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2.0 INTRODUCTION

Mononuclear phagocytes are characterized by extensive plasticity, wide-spread tissue distribution and an ability to respond to a wide range of environmental stimuli, most notably cytokines and microbial products (1-3). These endogenous and exogenous stimuli interact with a broad range of cell membrane receptors resulting in altered intracellular signaling and complex changes in gene activation and suppression. This, in turn, can lead to changes in a number of important physiological processes including cellular adhesion and migration, cytokine and chemokine production, antigen processing and host defense mechanisms (4-7). The identification of biomarkers that can be used to discriminate between these different modalities of mononuclear phagocyte activation would be highly beneficial for understanding the relationship between activation and various pathophysiological conditions.

By analogy to the T_H1/T_H2 nomenclature of $CD4^+$ helper T cells, it has been recently proposed that mononuclear phagocytes may also become polarized along either classical pro-inflammatory (M1) or alternative anti-inflammatory (M2) activation pathways (8-14). M1 cells are classical pro-inflammatory macrophages that express high levels of IL-1β, IL-12, IL-23 and TNF-α, produce effector molecules such as reactive oxygen and nitrogen intermediates, participate in the induction of polarized T_H1 responses and mediate resistance to tumors and intracellular pathogens. Monocyte-derived macrophages (MDM) can be induced to differentiate into M1 cells with GM-CSF or IFN-γ, either alone, or in combination with bacterial LPS or TNF-α (11, 15, 16). In contrast, M2 macrophages are anti-inflammatory cells that secrete IL-10, produce small amounts of IL-12 and IL-23, express high levels of scavenger (CD163) and mannose (CD206) receptors

(R) and participate in polarized T_H2 responses (11, 15, 17-19). M2 macrophages are generally considered to be more heterogeneous than M1 cells and, depending on their state of activation, can participate in a number of diverse activities aiming at suppressing inflammation, enhancing phagocytosis, promoting tissue repair and eliminating parasites (3, 11, 15, 17-23). In order to reflect these different forms of activation, M2 cells have been further subdivided into M2a, M2b and M2c subgroups (12). M2a (induced by exposure of macrophages to IL-4 or IL-13) and M2b (induced by stimulation with immune complexes, TLR or the IL-1R antagonist, IL1ra) cells exert immuno-regulatory functions and drive T_H2 responses, while M2c macrophages (generated by stimulation with IL-10) play a predominant role in the suppression of immune responses and tissue remodeling (12).

Transcriptome analyses conducted in mouse models, and more recently in human cells, have identified a large number of genes that are differentially expressed in M1 versus M2a macrophages. These studies have highlighted important interspecies differences in the gene expression profiles associated with the M1 and M2a polarization (14). In this study, I used a human monocyte-derived-macrophage (MDM) model to examine polarization-induced changes in MDM function and phenotype at the protein level. Some cell surface R were either up- or down-regulated in both M1 and M2a MDM relative to unpolarized controls, while other R were differentially modulated by M1 versus M2a polarization. One surface R, dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) was strongly upregulated in M2a- and downregulated in M1-MDM. Several cytokines and chemokines were differentially upregulated by M1 or M2a polarization. In most cases, secretion of these cytokines and chemokines returned to baseline levels 3 to 7 days post-polarization. In contrast, two chemokines, CXCL10 (M1) and CCL22 (M2a) remained upregulated for at least 7 days after exposure to a single polarizing stimulus. Further amplification of these signals was mediated by a transient down-regulation of cytokines and chemokines typically secreted by macrophages of the opposite activation

phenotype. Thus, M1 and M2a polarization programs appear to have a profound but transient effect on macrophages, altering their capacity to respond to signaling events involved in tissue homeostasis. Several of the markers described in this study may prove useful in assigning a functional role to infiltrating macrophages in damaged tissue.

2.1 MATERIALS AND METHODS

2.1.1 *Reagents.* Human recombinant cytokines and Lipid A (*Escherichia coli* F583) were purchased from R&D Systems (Minneapolis, MN) and used at concentrations of 2 ng/mL (TNF-α), 20 ng/mL (IFN-γ and IL-4) and 1 μg/mL (Lipid A). All cytokines were declared by the manufacturer to contain <0.1 ng/μg of contaminating LPS. Brefeldin A (BFA) and BSA were purchased from Sigma-Aldrich (St. Louis, MO). Ab were purchased from Immunotools (Friesoythe, Germany) unless otherwise specified. Ficoll-Hypaque was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). DMEM, PBS, FBS, normal human serum (NHS), penicillin, streptomycin, and glutamine were obtained from Cambrex (Verviers, Belgium).

2.1.2 *Isolation of monocytes and differentiation of human monocyte-derived macrophages (MDM).* PBMC were isolated from the buffy coat of healthy blood donors by Ficoll-Hypaque density gradient centrifugation. The cells were then washed, re-suspended in complete culture medium (D-MEM) supplemented with 1% pen/strep, 1% glutamine, 10% heat-inactivated FBS and 5% heat-inactivated NHS and seeded into 75 cm^2 flasks (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) at $8x10^6$ cells/mL. After 2 h of incubation at 37° C in a humidified atmosphere containing 5% CO_2 non-adherent cells were removed by gentle pipette aspiration; an equivalent volume of fresh complete medium was added to each flask. After 24 h of culture, adherent cells were washed twice with PBS, detached from the flask by scraping with a rubber policeman, and counted after Trypan blue vital dye staining. The cells (≥85% monocytes as determined by staining with anti-CD14 mAb and flow cytometric analysis) were seeded into 48-well plastic plates (Falcon) at $2.5x10^5$ cells/well and cultivated for 7-8 additional days at 37 °C in 5% CO_2 to promote their full differentiation in MDM (24). Seven-day-old MDM (≥95% $CD14^+$) were then stimulated with either IL-4 or TNF-α plus IFN-γ for 18 h in order to obtain M2a or M1 cells, respectively, as described (14). Following 18 h of cytokine stimulation, M1-,

M2a-, and control (unstimulated) MDM were washed with cytokine-free medium and further analyzed in parallel cultures.

2.1.3 *Flow cytometric analysis of cell surface determinants.* Adherent MDM were washed with cold EDTA/PBS (2 mM) and detached from the plastic plates by incubation with cold EDTA/PBS for 30 min at 4 °C and scraping with a rubber policeman. Non-specific binding of Ab was eliminated by pre-incubation of the cells in medium containing 15% serum (10% FBS and 5% NHS) for 15 min at 4 °C. Cells were then labeled by incubation with mAb for 30 min at 4 °C. Anti-CD18 (FITC), anti-CD4 (PE) and anti-HLA-DR (PE) were purchased from Immunotools (Immunotools, Friesoythe, Germany); anti-CXCR4 (PE) mAbs from Becton Dickinson (Frankline Lakes, New Jersey); anti-CD16 (PE) from Beckman Coulter (Miami, Florida) and anti-CD163 (PE) and anti-CD209 (PE) mAb were obtained from and R&D Systems. Cells were washed with cold PBS and fixed with 2% paraformaldehyde (PFA). CCR5 expression was assessed using biotinylated human CCL4 (Fluorokine, R&D Systems). Flow cytometric acquisition was performed by means of CYAN ADP flow cytometer (DAKO Cytomation, Glostrup, Denmark) and the results were analyzed with the FlowJo software version 8.4.3 (Tree Star Inc., Oregon).). Results are reported as percentage of positive cells and mean fluorescence intensity (MFI), the later describing the level of expression on positive cells.

2.1.4 *Cytokine and chemokine expression.* Commercially available ELISA kits (R&D Systems) were used to individually measure CCL3, CCL22 and CCL24 concentrations in MDM culture supernatants. Customized Bio-Plex plates (Bio-Rad, Hercules, CA) were used to quantify secretion levels of IL-1, IL-1ra, IL-6, IL-10, IL-12, CCL2, CCL4, CCL5 and CXCL10 according to the manufacturers' instructions.

Intracellular staining for CCL3 expression was determined after 18 h of polarizing cytokine stimulation in medium containing BFA (1 μg/ml). Following cytokine stimulation, M1- and M2a-MDM were detached from the plastic surface by scraping and fixed in 2% PFA. The cells were then permeabilized with Saponin buffer (0.1% Saponin,

0.5% BSA in PBS) and stained with Allophycocyanin conjugated anti-CCL3 Ab for 30 min at 4°C. After staining, the cells were washed twice with cold PBS and analyzed with a CYAN ADP apparatus (DAKO Cytomation).

2.1.5 *Statistical analysis.* Prism 5 from GraphPad Software (La Jolla, CA) was used for statistical analyses. Results are reported as mean values ± SD. The values were first normalized to those of unstimulated control cells and the analysis of variance followed by one-way ANOVA and the Tukey post-test. To compensate for inter-donor variability, all assays were performed on triplicate samples with MDM derived from 4 to 6 independent donors, as further specified.

2.2 RESULTS

2.2.1 *Cytokine polarization shapes MDM morphology.* After 7 days of differentiation, prior to stimulation with either TNF-α plus IFN-γ (M1) or IL-4 (M2a), MDM exhibited a mixed morphology consisting of approximately equal numbers of spindle-shaped fibroblastoid and large flat-round/fried egg-shaped cells (50.5±6.2 and 49.56.1%, n=5), respectively (**Figures 2.1 A, left panel**). Stimulation for 18 h with M1 polarizing cytokines led to a significant increase in the number of fibroblastoid cells (69±6.2%, n=5, p=0.027 vs. controls; **Figure 2.1 B**) and in the length of the cellular projections extending from the cell poles (**Figure 2.1 A, middle panel**). Conversely, IL-4 stimulation of MDM was associated with a significant increase in the number of flat-round cells (73±14.1%, n=5, p=0.016 vs. controls; **Figure 2.1 B**). When compared to control MDM, M2a cells exhibited a more condensed morphology with short cellular projections accumulating at the poles of the cells (**Figure 2.1 A, right panel**). Activation had no adverse effect on cell survival as assessed by Trypan blue dye exclusion.

2.2.2 *Differential expression of cell surface molecules by M1- and M2a-MDM.* Recent transcriptional profiling has led to the identification of more than 2,000 genes that are regulated by M1 or M2a polarization (14). Although highly informative, it is well known that the levels of a given RNA transcript do not always correlate with the amount of protein that is synthesized and, eventually, secreted (25). In this study, I investigated and quantified changes in protein expression in response to M1 vs. M2a polarization, focusing on cell surface determinants that are known to play a key role in immune modulation and HIV pathogenesis. Some molecules, such as the integrin β chain CD18, were highly expressed in control MDM and were unaffected by either M1 or M2a polarization both in terms of the percentage of positive cells and their mean fluorescence intensity (MFI; **Figure 2.2** and **Table 1**). Other surface molecules showed a similar increase (or decrease) in both M1- and M2a-MDM. HLA-DR, which was highly expressed in control MDM, was further upregulated in terms of MFI in both M1- and M2a-MDM (**Figure 2.2** and **Table 1**).

A significant decrease in the percentage of cells expressing CD4 and CXCR4 occurred in M1 and, to a lesser extent, in M2a cells (**Figure 2.2** and **Table 1**). In contrast, expression of CCR5, remained unchanged and, in some donors, was upregulated on the surface of M2a-MDM vs. M1- and controls (**Figure 2.2 and Table 1**). These surface molecules serve as the main entry R (CD4) and co-R (CXCR4, CCR5) for HIV-1, the causative agent of AIDS (26-28). The biological implications of HIV-1 R modulation will be further discussed in Chapter 3.

Other surface R were differentially regulated by cytokine polarization. The FcγRIII (CD16) and the hemoglobin scavenger (CD163) R were clearly expressed on control MDM (**Figure 2.2**). Exposure to M1 cytokines significantly decreased the number of both $CD16^+$ and $CD163^+$ cells in association with the disappearance of a brightly stained population of cells bearing the CD163 molecule (**Figure 2.2**). M1 stimulation also resulted in a down-regulation in the number of these R on a per cell basis, especially in the case of $CD16^+$ (**Table 1**). Polarization towards M2a-MDM, on the other hand, had no effect on either the number of positive cells, or level of CD16 or CD163 expression relative to control MDM (**Figure 2.2** and **Table 1**).

2.2.3 *DC-SIGN is selectively upregulated in M2a-MDM and downregulated in M1-MDM.* DC-SIGN, a molecule that plays a crucial role in capturing viral pathogens such as HIV and hepatitis C virus (HCV) (29-32) on the surface of myeloid DC (33), was expressed at low levels on the surface of control MDM (**Figure 2.2**). A near complete disappearance of DC-SIGN was observed in M1-MDM. This is in sharp contrast to the upregulation of this receptor on M2a cells, both in terms of percentage of positive cells and MFI (**Figure 2.2** and **2.3**, and **Table 1**).

2.2.4 *Differential patterns of chemokine and cytokine secretion by M1- and M2a-MDM.* The impact of M1- and M2a-MDM polarization on the secretion of different chemokines (CCL2, CCL3, CCL4, CCL5, CCL22, and CXCL10), pro-inflammatory (IL-1β, IL-6, IL-12) and anti-inflammatory (IL-1ra, IL-10) cytokines was evaluated in cell

culture supernatants collected at regular intervals post-stimulation. After 18 h of differential stimulation, a significant upregulation of CXCL10, CCL3 and CCL4 was observed in the supernatants of M1-MDM relative to M2a-MDM (215 vs. 2.5 fold, 25 vs. 2.2 fold, and 14 vs. 1.6 respectively; **Table 2**). CCL5, which was undetectable in both control and M2a-MDM, was significantly upregulated in M1-MDM in 50% of donors (**Table 2**). M2a-MDM, on the other hand, showed a 3-fold increase in CCL22 and a relatively modest but significant 2-fold reduction in CCL2 production.

In order to determine whether activation induced polarization affected the entire MDM population, or only a cell subset of these cells, the synthesis of the M1-induced chemokine, CCL3, was measured at the intracellular level using a fluorescence-labeled anti-CCL3 Ab. Although no changes were detected in the MFI of $CCL3^+$ M1-MDM versus control MDM, a clear increase in the percent of CCL3+ cells was observed in M1- relative to control MDM (ie. from 1-3% to 12-21%, a mean increase of 7 ± 2.31-fold vs. Nil)($n = 5$, $p=0.023$; **Figure 2.4 A**). Stimulation with Lipid A resulted in an even greater (20-fold) increase in both the number and MFI of MDM expressing intracellular CCL3 (**Figure 2.4**) suggesting that M1 polarization affects only a subset of the macrophages with the potential to secrete CCL3. Compared to chemokine secretion, the impact of M1 and M2a polarization on cytokine production was quantitatively less dramatic. A 2.3-fold increase in IL-6 and a 5.2-fold increase in IL-10 secretion was observed after 18 h of polarization with M1 and M2a cytokines, respectively, while no effects were observed on either IL-12, IL-1β or IL-1ra secretion (**Table 2**).

2.2.5 *Transient nature of M1-MDM and M2a-MDM functional polarization.* To examine the duration of the stimulatory response, MDM were polarized for 18 h toward either an M1 or M2a phenotype, and then incubated for an additional 7 days in fresh cytokine-free complete medium. Removal of the stimulatory cytokines led to a near complete reversal of M1- and M2a- induced morphologies. These morphological changes were associated with a parallel reversion in M1 and M2a surface R phenotypes (data in

chapter 3 and 4). Most secretory molecules, including CCL3, IL-6 and IL-10, returned to baseline levels 3 to 7 days after removal of the polarizing cytokines (**Figure 2.5**). In contrast, CXCL10 secretion remained significantly (p=0.004) upregulated for at least 7 days in M1-MDM compared to control cultures. Furthermore, secretion of CCL22, a chemokine upregulated in M2a-MDM, was further upregulated after the removal of IL-4 (p=0.032). CCL22 expression remained above control levels until day 7, the last time point studied (**Figure 2.5**).

2.2.6 *Contra-regulatory effects of M1/M2a polarization on cytokine and chemokine expression.* In addition to the fading of most M1- and M2a-MDM features, removal of the polarizing cytokines was associated with an impaired secretion of cytokines and chemokines associated with the opposite polarization pathway. In particular, secretion of the M1-associated factors CCL3, CXCL10 and IL-6 was significantly, although transiently, decreased in M2a cultures following removal of IL-4. Conversely, secretion of the M2a-associated molecules CCL22, IL-10, and IL-1ra was decreased in M1-MDM (**Figure 2.6**). Thus, although transient, the polarized secretion of M1 and M2a cytokines and chemokines is amplified by a secondary wave of down modulation involving molecules of the opposite activation pattern.

2.3 DISCUSSION

In this study, I investigated the phenotypic and functional properties of differentially polarized human MDM obtained after 18h of stimulation with TNF-α plus IFN-γ (M1-MDM), or IL-4 (M2a-MDM), respectively. M1-MDM assumed a predominantly fibroblastoid shape while M2a-MDM were mostly flat-round/fried egg-shaped. Certain cell surface R, such as CD16 (FcγIIIR) and CD163 (hemoglobin scavenger R), were differentially regulated in M1- vs. M2a-MDM, whereas others were concordantly up- (CCR5, HLA-DR) or down- (CD4, CXCR4) modulated in both M1 and M2a-MDM. DC-SIGN was the only determinant showing a clear-cut, reciprocal pattern of downregulation in M1- and upregulation in M2a-MDM. Polarization-induced changes in chemokine expression were more dramatic than those observed for cytokines. CCL3 and CXCL10 were upregulated in M1-MDM. CCL22 was upregulated and CCL2 was downregulated in M2a-MDM. Most phenotypic and functional changes were transient with full reversion occurring 3-7 days after removal of the activating stimulus. The two exceptions were CCL22 (M2a) and CXCL10 (M1) which remained upregulated for at least 7 days post-stimulation. Finally, a "mirror image" downregulation of M1 cytokines and chemokines in M2a-MDM and of M2a cytokines and chemokines in M1-MDM was noted with trough levels occurring 1-3 days after removal of the polarizing cytokines.

The finding that M1-MDM were mostly fibroblastoid-like, whereas M2a-MDM were primarily flat-round, egg-shaped cells is consistent with previous studies (20). However, it has also been reported that exposure of MDM to TGF-β, a type 2 cytokine with anti-inflammatory properties, induces a fibroblastoid-like morphology similar to M1 macrophages (20). While correlated to M1 and M2a polarization, this suggests there is no absolute associated between morphology and inflammatory and ant-inflammatory profiles.

Of particular note, was the clear-cut upregulation of DC-SIGN on M2a-MDM. This finding is in sharp contrast to its downregulation on M1-MDM and its low-level expression on control macrophages. These results are compatible with transcriptome studies showing

a 27-fold increase of DC-SIGN mRNA in M2a- relative to M1-MDM (14). DC-SIGN+ DC have been shown to secrete TNF-α and IL-6 in response to LPS (37), stimulate the proliferation of allogeneic T-cells and capture, store and effectively transmit HIV-1 to $CD4^+$T lymphocytes *in trans* (38). My observations suggest that DC-SIGN+M2a-MDM may exhibit similar functions including the ability to transmit HIV-1 *in trans* (see Chapter 4). When expressed on alveolar macrophages and Kupffer cells, DC-SIGN may also contribute to the establishment and dissemination of diseases such as Ebola, Marburg and SARS or HCV respectively (30, 31, 39, 40).

CD16 (FcγRIII) and CD163 (hemoglobin scavenger) R were expressed at high levels on control and M2a-MDM, but not on M1-MDM. Upregulation of CD16 on human monocytes stimulated with GM-CSF, IL-4 and IL-10, and its downregulation by TGF-β, have been previously reported (41). In mice it has been reported that CD16 and other FcR, such as CD32 and CD64, are increased on the surface of M1, but not M2a, macrophages further indicating that there are important differences between the murine and human systems (12). Interestingly, CD16+ monocytes are increased in several inflammatory diseases and in the central nervous system increased infiltration and differentiation of HIV-1-infected CD16+ monocytes into perivascular macrophages is associated with development AIDS-related dementia.

Expression of HLA-DR, a prototypic marker of immunological activation, was increased on both M1 and M2a cells with a greater increase occurring in M1-MDM. These results are consistent with published data showing that IFN-γ upregulates HLA-DR in most macrophages, while IL-4 increases HLA-DR expression on a subset of macrophages only (18, 42). MDM stimulated with IFN-γ have also been shown to express higher steady state levels of HLA-DR mRNA and surface Ag expression compared to cells stimulated with IL-4 (42).

CCR5 and CXCR4, the main HIV-1 entry co-receptors (26, 44), were also modulated by polarization. Similar to HLA-DR, CCR5 was upregulated, in some donors,

by M2a activation. Although CCR5 is well-recognized marker of T cell activation, regulation of its expression on macrophages is not well defined. Some studies have suggested that IFN-γ upregulates CCR5 on adult blood monocytes (45), while others have reported downregulatory effects (46). In contrast to CCR5, CXCR4 was downregulated on both M1- and M2a-MDM. These results differ from studies conducted in T cells (47-49) and from a recent transcriptional study showing that, in humans, M1 polarization leads to an upregulation of CXCR4 mRNA (14). However, our results are is in agreement with publications showing that, at the protein level, both IFN-γ and IL-4 decrease CXCR4 expression (46, 50).

In agreement with transcriptome studies (14), differential regulation of chemokine secretion was a dominant feature of M1 and M2a polarization of human MDM. A major, and consistent, upregulation of CCL3 (MIP-1α) and CCL4 (MIP-1β) and an even greater increase in CXCL10 (Interferon Inducible Protein-10, IP-10) was observed in M1-MDM vs. control and M2a-MDM. Secretion of these chemokines has been linked to the recruitment of activated Th1 cells, NK cells, and mononuclear phagocytes expressing either CCR1 and CCR5, or CXCR3, respectively (12). CCL22 (Macrophage Derived Chemokine, MDC), a chemokine that was constitutively expressed before polarization, was even further up-regulated in M2a- relative to M1- and unstimulated MDM. The failure to super-induce CCL22 expression in M1-MDM is consistent with the known inhibitory effect of IFN-γ on CCL22 expression (5, 12). Another chemokine constitutively expressed by MDM, CCL2 (MCP-1) was downregulated in M2a-MDM vs. control cells (**Table 2**). This chemokine plays a central role in the recruitment of monocytes to sites of tissue inflammation. The impact of M1/M2a polarization on cytokine secretion was less quantitatively important than that observed for chemokines. However, the patterns observed confirmed the pro- and anti-inflammatory commitments of the M1 and M2a activation programs.

A recurrent finding was the significant overlap in expression patterns between control and M2a-induced markers. In this regard, it has been suggested that the M2 activation may represent a default phenotype that serves to maintain a balanced microenvironment in the lung, gut and other tissues that are under constant microbial stimulation (11, 14). Alternatively, circulating monocytes may be predisposed toward a M2 phenotype as a result of exposure to relatively high levels of M-CSF in plasma (from 187-7,604 pg/ml) (51). This hypothesis, suggested by Martinez et al. (14), was based on transcriptional data showing that M1 polarization is associated with more dramatic variations in steady-state mRNA relative to M2a polarization (90% vs. 8% variance from the control, respectively).

Along with morphological heterogeneity, some cell surface markers, including CD16, CD163, HLA-DR, CCR5 and DC-SIGN, showed a heterogeneous bimodal distribution pattern when analyzed by flow cytometry. Heterogeneity within our MDM population was further confirmed in experiments showing differences in the number of M1-MDM expressing intracellular CCL3 after activation with IFN-γ plus TNF-α versus Lipid A, a stimulus that acts via the CD14/TLR4 complex, to up-regulate CCL3 expression (52). (15% and >80%, respectively). These findings suggest that IFN-γ plus TNF-α stimulates only a subset of MDM that are capable of producing CCL3. The reasons why our MDM appear more heterogeneous than in another recent study are unclear (20), but may be due to differences in culture conditions, the type of stimulus, and (or) the duration of the activation period. Our polarization protocol was adopted and modified from previous studies conducted with human cells (14). However, our monocytes were differentiated in the presence of human serum rather than GM-CSF and/or M-CSF (24). It has been suggested that these two cytokines can pre-bias MDM toward either M1 (GM-CSF) or M2 (M-CSF) pathways, reducing the inherent heterogeneity of the MDM population (3, 11, 14, 53).

My study also yielded new information on the kinetics of M1/M2a polarization and its reversibility. Similar to other studies (14), most polarization-induced changes were detected after only 18 h of polarization confirming the ability of macrophages to rapidly respond to a range of different inflammatory and anti-inflammatory signals. In contrast to most activation markers, which showed a rapid return to baseline within the first 3 days, secretion of CXCL10 and CCL22 remained persistently elevated for at least 7 days after removal of the polarizing signals, in M1- and M2a-MDM, respectively. These findings suggest that some functional aspects of M1 and M2a polarization are relatively long-lasting despite removal of the inductive signal. In this regard, I also detected a symmetrical wave of counter modulatory effects leading to the downregulation of M1-associated cytokines and chemokines in M2a-MDM, and of M2a cytokines/chemokines in M1-MDM. These effects included the downregulation of IL-10, IL-1ra and CCL22 secretion in M1-MDM, and of IL-6, CXCL10 (IP-10) and CCL3 (MIP-1a) in M2a-MDM as well as downmodulation of CD4 and CXCR4 in both polarizing conditions. These observations are consistent with studies describing the inhibitory effects of IL-4 (an M2/Th2-inducing cytokine) on the M1-associated chemokines CCL5 (RANTES), CXCL9 and CXCL10 (IP-10) (12).

In summary, I have identified a discrete panel of protein biomarkers that can be used to investigate and accurately discriminate between pro-inflammatory M1 (DC-SIGN^{neg},CXCL10, CCL3, IL-6) and anti-inflammatory M2a (DC-SIGN^{+}, CCL22, IL-10) macrophages in humans. These markers should prove useful for investigating the impact of different tissue environments on macrophage phenotype and function, and for elucidating the contribution of macrophages to a wide range of pathological conditions, including cancer and infectious diseases.

2.4 TABLES

Table 1. Cell surface phenotype changes (percent) in M1- and M2a- vs. control MDM.

Marker	N°	Parameter	M1	M2a	M1 vs. Control	M2a vs. Control	M1 vs. M2a
CD18	4	positive cells	0.87±0.39	1.14±0.05			
		MFI	1.00±0.16	1.19±0.27			
CD4	5	positive cells	0.14±0.08	0.32±0.08	**	**	*
		MFI	0.92±0.07	0.91±0.05	*	*	
CXCR4	5	positive cells	0.28±0.23	0.50±0.09	*	**	
		MFI	1.13±0.29	1.14±0.21			
CCR5	5	positive cells	1.27±0.06	1.87±0.72			
		MFI	0.98±0.01	0.91±0.11			
HLA-DR	4	positive cells	1.07±0.06	1.00±0.72	*		*
		MFI	1.89±0.01	1.57±0.11	*	*	
CD16	4	positive cells	0.09±0.11	0.92±0.13	**		*
		MFI	0.34±0.41	0.94±0.15	*		
CD163	4	positive cells	0.57±0.10	0.98±0.02	*		*
		MFI	0.42±0.10	0.92±0.10	*		**
DC-SIGN	4	positive cells	0.34±0.11	9.08±2.93	*	*	*
		MFI	0.74±0.25	2.11±1.11		*	*

^Fold changes vs. control MDM. Expression of surface antigens was analyzed by cytofluorimetric assay. *p≤0.05,** p≤0.001 as assessed by one way ANOVA and tukey post test. °N identifies the number of independent monocyte donors.

Table 2. Cytokine and chemokine secretion changes (percent) in M1- and M2a- vs. Control MDM.

Chemokine/ Cytokine	N°	Range (Control) (pg/ml)	Mean° ±SD	M1 (fold vs. control)	M2a (fold vs. control)	M1 vs. Control	M2a vs. Control	M1 vs. M2a
CCL2	4	2,519 - 8,506	5,006±2,783	1.4±0.6	0.5±0.3		*	*
CCL3	6	26 - 274	82.5±96	25±8.3	2.2±1.1	*	*	*
CCL4	6	154-4,532	222±58	14±5.4	1.6±0.8	**		**
CCL5	6	^n.d	^n.d	69±85.8	^n.d			
CCL22	4	165-886	398±330	1±0.4	3.9±0.3		*	*
CXCL10	4	185 – 6,186	1,734±2526	215±157	2.5±3.3	*		*
IL-1β	4	0.17 - 0.28	0.21±0.04	1.1±0.5	1.1±0.3			
IL-1ra	6	115 – 3,031	1,572±1458	1.0±0.3	0.9±0.3			
IL-6	4	0.38 - 18	6.9±7.6	2.3±1.1	1.0±0.3	*		*
IL-10	6	0.99- 18	6.2±6.9	1.7±0.7	5.2±3.5	*	*	*

Chemokines and cytokines were tested after 18 h of polarizing stimulation of MDM. * $p \leq 0.05$ as assessed by one way ANOVA and tukey post test. ^n.d = not detectable

2.5 FIGURE LEGENDS

Figure 2.1 Morphology of polarized and unpolarized MDM cultures. A. Monocytes were differentiated for 7 days in complete medium containing human serum and then cultivated for additional 18h in the same medium (Control), or in medium containing M1 or M2a inducing cytokines (Original magnification: 40X). **B.** Mean percentage of fibroblastoid vs. flat-round cells in M1- and M2a-MDM established from the PBMC of 5 independent healthy donors. *p $\leq$0.05 (M1 vs. M2a vs. Control for both morphological types). Percentages were determined by counting the number of fibroblastoid and flat-round cells from 3 individual fields per each culture condition from MDM of every donor.

Figure 2.2 Cell surface determinants of M1- and M2a-MDM. The results shown refer to the analysis of MDM derived from monocytes of a single donor representative of 5 donors tested. Unstimulated MDM and MDM activated for 18 h with M1 or M2a cytokines were labeled with a panel of mAb and analyzed using Cyan ADP flow cytometer and FlowJo software.

Figure 2.3 Differential expression of DC-SIGN on M1- and M2a-MDM. MDM were stained with a PE conjugated mAb directed against DC-SIGN. Results of the MDM cultures established from 4 independent donors are expressed as the average fold induction/reduction relative to their unpolarized control cells.

Figure 2.4 Upregulation of CCL3 expression in a subset of M1-MDM. A. Intracellular levels of CCL3 in control (Nil) MDM, and in MDM stimulated with M1 or M2a polarizing cytokines or with Lipid A. Results from MDM cultures established from monocytes of a single donor representative of five tested are shown. **B.** Percentage and MFI of CCL3 expressing cells in control MDM (Nil) and in MDM subjected to different polarizing conditions. MDM were established from monocytes of 5 independent healthy donors. *p $\leq$0.05 (M1 vs. M2 vs. Nil)

Figure 2.5 Transient modulation of cytokine and chemokine secretion in M1- and M2a-MDM. M1- and M2a-MDM were washed and cultivated for 7 days in complete cytokine-free medium. In order to normalize inter-donor variability and differences in secretion levels among different analytes, all results were expressed in terms of fold induction/reduction relative to autologous control cultures. Levels of cytokines were assessed using custom-made Bio-Plex plates (IL-1ra, IL-6, IL-10, CXCL10) or commercially available ELISA assays (CCL3, CCL22). The bar graphs show the average result for 4-6 donors per cytokine, and are expressed as the fold induction relative to control MDM (*$p \leq 0.05$, one way ANOVA).

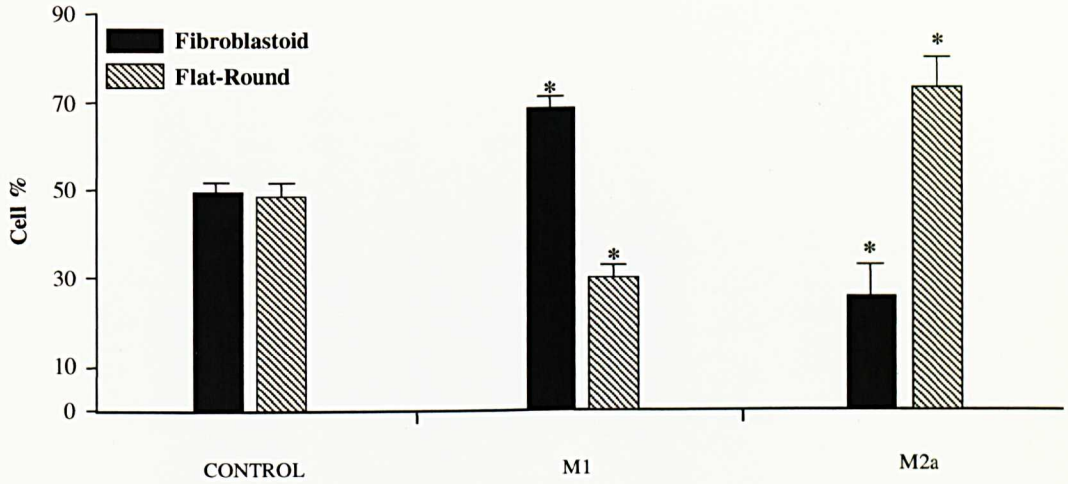
Figure 2.6 Transient downregulation of opposing chemokines/cytokines following M1 and M2a activation. After removal of the stimulatory cytokines a transient down-regulation of M2a-related cytokines and chemokines was commonly observed in M1 stimulated MDM, and of M1-related cytokines/chemokines in M2a-MDM. Results are expressed as the average of the mean fold induction of 4-6 donors per secreted chemokine/cytokine. Statistical differences were assessed using one way ANOVA and tukey post-test.

2.6 FIGURES

Figure 2.1

A.

B.



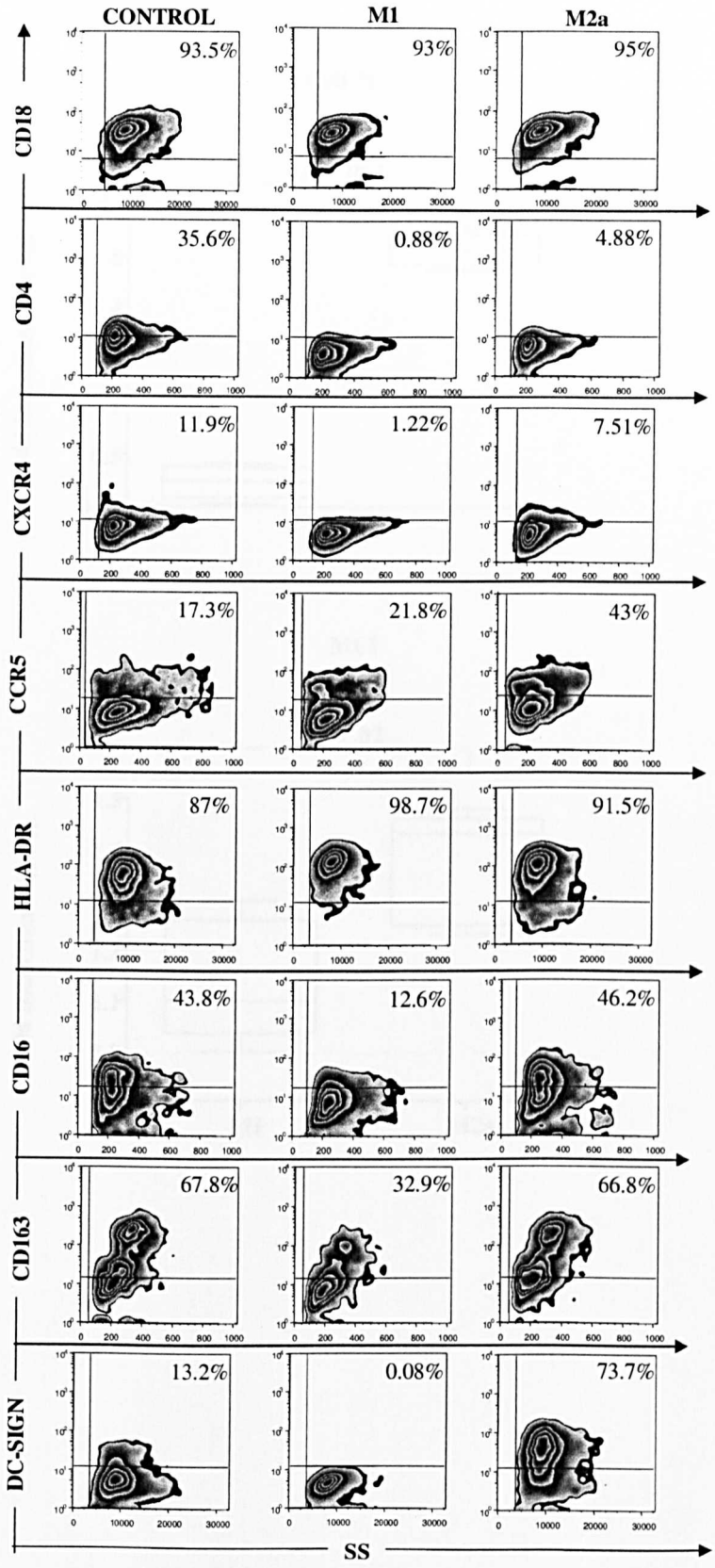

CONTROL
M1
M2a
CD18
93.5%
93%
95%
CD4
35.6%
0.88%
4.88%
CXCR4
11.9%
1.22%
7.51%
CCR5
17.3%
21.8%
43%
HLA-DR
87%
98.7%
91.5%
CD16
43.8%
12.6%
46.2%
CD163
67.8%
32.9%
66.8%
DC-SIGN
13.2%
0.08%
73.7%
SS

Figure 2.2

Figure 2.3

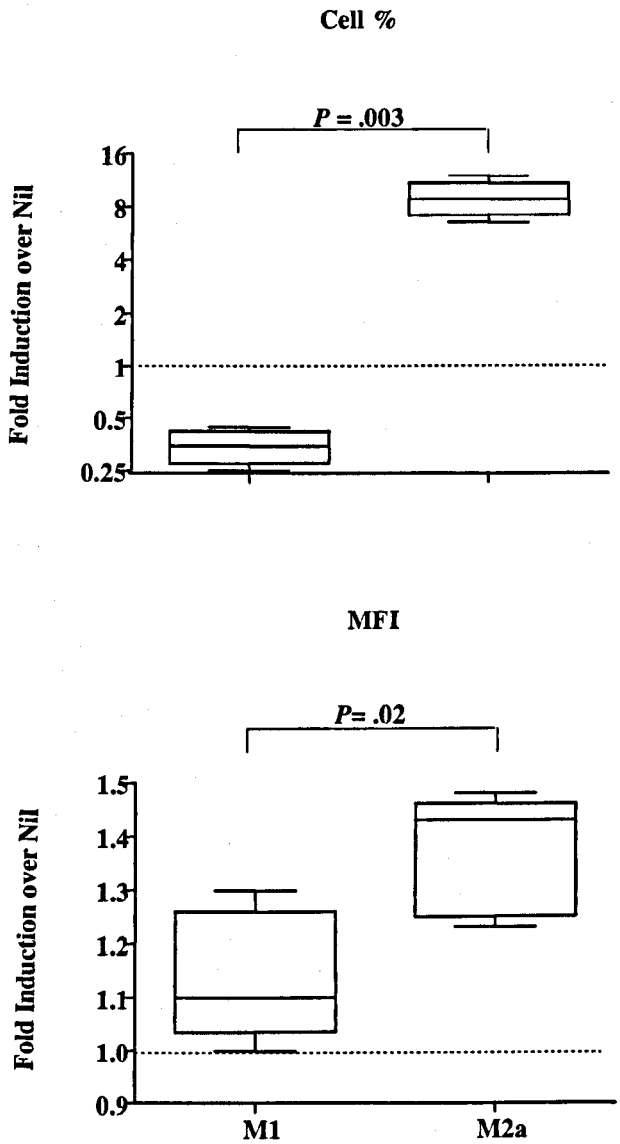
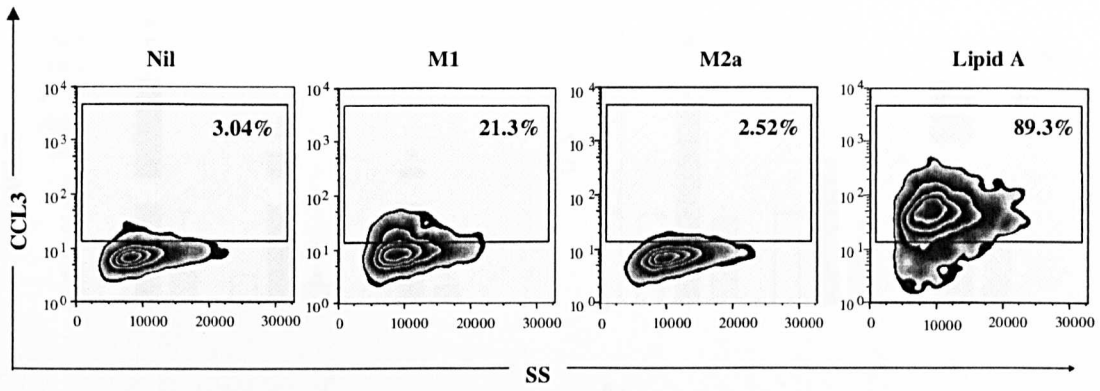

Cell %
P = .003
Fold Induction over Nil
16
8
4
2
1
0.5
0.25
MFI
P= .02
Fold Induction over Nil
1.5
1.4
1.3
1.2
1.1
1.0
0.9
M1
M2a

Figure 2.4

A.


Nil
M1
M2a
Lipid A
3.04%
21.3%
2.52%
89.3%
CCL3
SS

B.

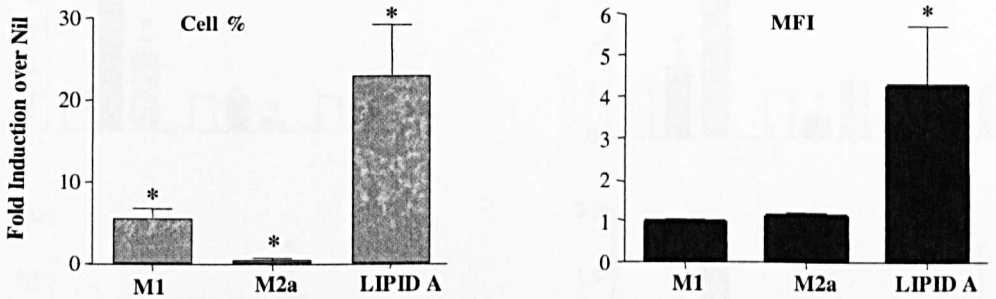

Fold Induction over Nil
Cell %
MFI
M1
M2a
LIPID A

Figure 2.5

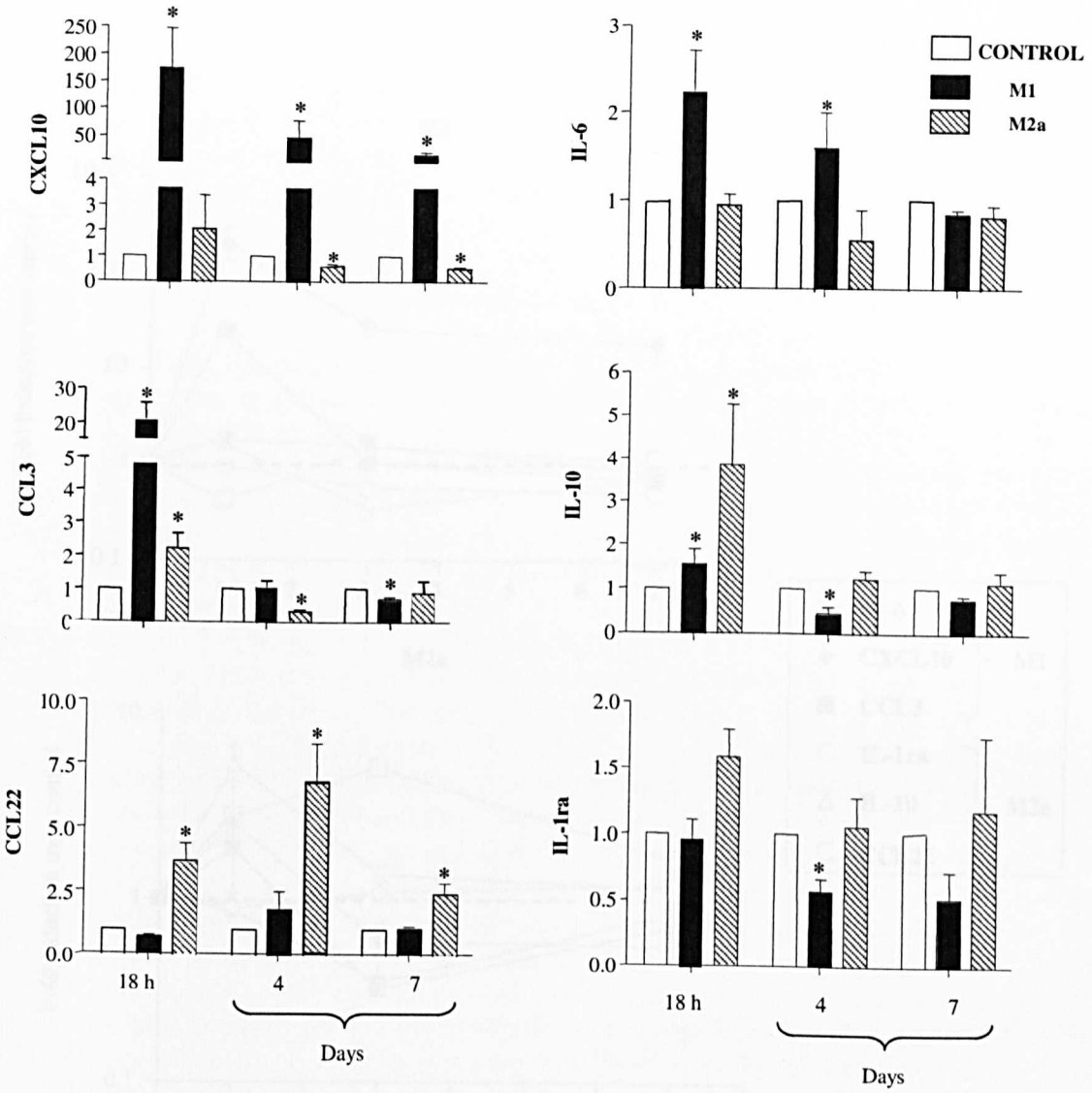
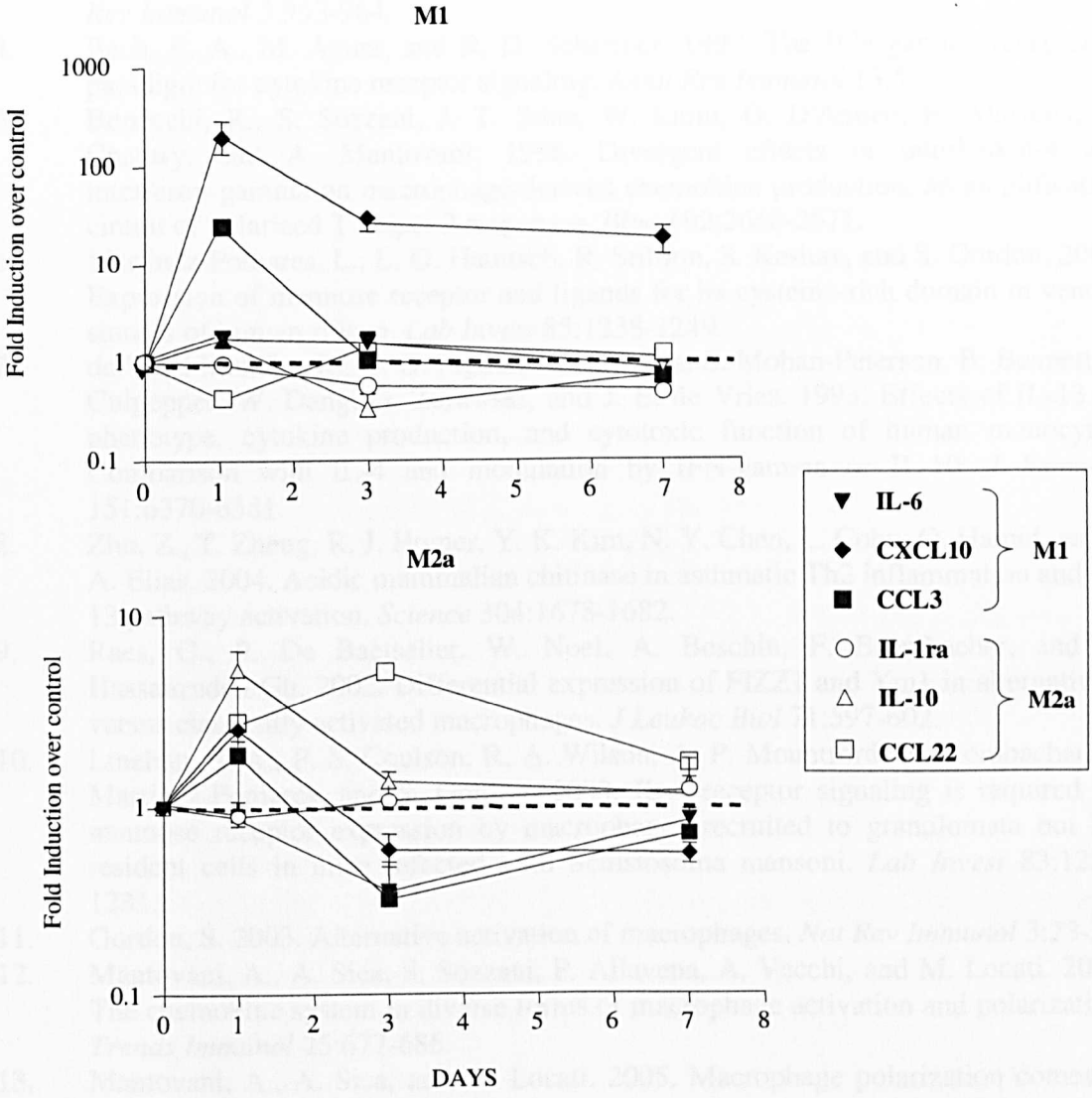


Figure 2.6


M1
Fold Induction over control
1000
100
10
1
0.1
0
1
2
3
4
5
6
7
8
M2a
Fold Induction over control
10
1
0.1
0
1
2
3
4
5
6
7
8
DAYS
IL-6
CXCL10
CCL3
M1
IL-1ra
IL-10
CCL22
M2a

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CHAPTER 3
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3.0 INTRODUCTION

Cells of the macrophage lineage play a pivotal role in a broad range of innate and adaptive immune responses. In addition, they are an important early target cell for human immunodeficiency virus type 1 (HIV-1) and contribute to the dissemination and cell-to-cell spread of the virus (1-4). Unlike $CD4^+$ T cells, macrophages are less refractory to the cytotoxic effects of HIV-1 and because of their longevity and ubiquitous distribution in tissues and organs serve as viral reservoirs throughout the course of HIV-1 disease (3-5). The early establishment of viral reservoirs during primary infection (6-8) has stimulated interest in characterizing the mechanisms underlying the persistence and replication of HIV-1 in both $CD4^+$ T cells and monocyte/macrophages (9, 10).

Macrophages respond to a variety of different cytokines and microbial gene products and their susceptibility to HIV-1 is profoundly influenced by these stimuli (11-16). Interferon-α/–β (IFN-α/–β) potently inhibit HIV-1 replication in macrophages while other cytokines, including interleukin-1 (IL-1) and IL-6 stimulate viral replication (17-19). Depending on the stage of macrophage differentiation and the timing of cytokine exposure, some cytokines can either promote or inhibit HIV-1 infection. IL-4 and IL-13, for example, enhance viral replication in monocytes, but inhibit replication in differentiated monocyte-derived macrophages (MDM) (20, 21). On the other hand, exposure of MDM to tumor necrosis factor-α (TNF-α) before infection is inhibitory, while stimulation of latently infected macrophages or monocytic cell lines leads to the upregulation of virus transcription and expression via activation of the cellular transcription factor NF-kB (16, 22, 23).

A relatively recent concept in immunology, developed primarily in mouse models, has been the observation that cytokine stimulation can trigger the polarization of

macrophages into opposing pro- and anti-inflammatory programs. By analogy with $CD4^+$ T helper (T_H) cell differentiation, these polarization pathways have been designated M1 and M2 (24-26). M2 macrophages are generally considered to be more heterogeneous than M1 cells. To reflect these differences M2 cells have been further subdivided into M2a, M2b and M2c subgroups (24). M2a (induced by exposure to IL-4 or IL-13) and M2b (induced by stimulation with immune complexes, TLR or the IL-1R antagonist, IL1ra) macrophages exert immunoregulatory functions and drive T_H2 responses, while M2c cells (generated by stimulation with IL-10) play a predominant role in the suppression of immune responses and tissue remodeling (24).

Recent molecular profiling of human MDM has identified >2,000 transcripts that are differentially modulated during M1 vs. M2a polarization giving rise to macrophages with highly specialized phenotypic and functional properties (27). Although it is well documented that cytokines play a critical role in the regulation of HIV-1 replication (28), the effects of M1 and M2 polarization on viral replication have not been thoroughly investigated. In this study, I investigated the effects of M1 (TNF-α plus IFN-γ) and M2a (IL-4) polarization on the ability of human MDM to support productive HIV-1 infection. The study focuses primarily on M1 and M2a cells since these phenotypes mediate T_H1 and T_H2 responses and it has been proposed that a switch from a T_H1 to a T_H2 immune response may play a role in the pathogenesis of HIV-1/AIDS (29, 30). I found that both polarization pathways led to a strong inhibition of virus production in association with specific alterations in the expression of cell surface receptors, chemokines and cytokines involved in HIV-1 entry, replication and dissemination. However, the magnitude and duration of the inhibitory response and the mechanisms underlying these responses were clearly different in M1- versus M2-MDM. This study describes the transient and reversible nature of these differential responses both in terms of cytokine/chemokine secretion and the cells' capacity to support productive infection.

3.1 MATERIALS AND METHODS

3.1.1 *Reagents.* Human recombinant cytokines were purchased from R&D Systems (Minneapolis, MN) and used at concentrations of 2 ng/mL (TNF-α) and 20 ng/mL (IFN-γ and IL-4), respectively. All cytokines were declared by the manufacturer to contain <0.1 ng of LPS per μg of protein. Ficoll-Hypaque was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). D-MEM, PBS, FBS, normal human serum (NHS), penicillin, streptomycin and glutamine were obtained from Cambrex (Verviers, Belgium).

3.1.2 *Isolation of human monocytes and their differentiation into monocyte-derived- macrophages (MDM).* Peripheral blood mononuclear cells (PBMC) were isolated from the buffy coats of healthy HIV-seronegative blood donors by Ficoll-Hypaque density gradient centrifugation. The cells were then washed, resuspended in D-MEM containing pen/strep (1%), glutamine (1%), heat-inactivated fetal bovine serum (FBS, 10%) and heat-inactivated normal human serum (NHS, 5%) (complete medium) and seeded into 75 cm^2 flasks (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) at of $8x10^6$ cells/mL. Non-adherent cells, including T lymphocytes, were removed by gentle pipette aspiration after 2 h of incubation at 37° C in a humidified atmosphere containing 5% CO_2, and an equivalent volume of fresh complete medium was added to each flask. After 24 h of culture, adherent cells were washed twice with PBS, detached from the flask by scraping with a rubber policeman, and counted after Trypan blue vital dye staining. These cells (≥85% monocytes as determined by flow cytometric analysis after staining with anti-CD14 mAb) were seeded into 48-well plastic plates (Falcon) at the concentration of $2.5x10^5$ cells/well and were cultivated for 7-8 additional days at 37 °C in 5% CO_2 to promote their full differentiation into MDM (12). These cells (≥95% $CD14^+$) were then stimulated for 18 h with either TNF-α plus IFN-γ or IL-4 in order to obtain M1- or M2a-MDM, respectively, as described (27). M1-, M2a- and unstimulated/unpolarized (control) MDM maintained in

culture for 18 h in the absence of specific cytokines or polarizing stimuli were washed with cytokine-free medium and further analyzed in parallel cultures.

In order to investigate the persistence of MDM polarization, M1-, M2a- and control MDM were thoroughly washed and cultivated for 7 additional days in the absence of further stimulation and monitored for cell surface antigen expression and cytokine/chemokine secretion after 0, 3 and 7 days post-polarization.

3.1.3 *Flow cytometric analysis of cell surface determinants.* Adherent MDM were washed with cold EDTA/PBS (2 mM) and detached from the plastic plates by incubation with cold EDTA/PBS (2 mM) for 30 min at 4 °C and scraping with a rubber policeman. Non-specific binding of Ab was eliminated by pre-incubating the cells in medium containing 10% FBS and 5% NHS for 15 min at 4 °C. The cells were then incubated with anti-CD4 (PE) (Immunotools, Friesoythe, Germany) monoclonal Abs (mAbs) for 30 min at 4°C, washed with cold PBS and fixed with 2% paraformaldehyde (PFA). CCR5 expression was assessed using biotinylated human CCL4 (Fluorokine, R&D Systems). Flow cytometry was performed using a CYAN ADP instrument (DAKO Cytomation, Glostrup, Denmark) and the results were analyzed with the FlowJo software version 8.4.3 (Tree Star Inc., Ashland, OR). Results are reported as percentage of positive cells and mean fluorescence intensity (MFI), the later being used to describe the level of expression on a population of positive cells.

3.1.4 *Beta-Chemokine secretion and expression.* Chemokines were quantified in culture supernatants of MDM 18 h after their polarization (day 0) as well as on days 3 and 7 post-polarization. The levels of CCL3 released into the supernatants of MDM cultures were quantified by commercial ELISA kits (R&D Systems), whereas the concentrations of CCL4 and CCL5 were determined in customized Bio-Plex plates (Bio-Rad, Hercules, CA). These assays were performed according to the manufacturer's instructions.

3.1.5 *HIV-1 infection and replication.* Control, M1-, and M2a-MDM were infected with the macrophage-tropic, CCR5-dependent (R5) laboratory-adapted strain HIV-1_{BaL} at

the multiplicity of infection (m.o.i) of 0.1. In certain experiments, MDM were also infected with HIV-1_{BaL} (m.o.i.=0.1) 3 and 7 days after polarization. To assess virus production and spread, multiple aliquots of the culture supernatant were collected every 3-4 days over a 5-week period and stored at -20° C. At the end of each culture period, these supernatants were thawed and analyzed for virus replication by measuring the levels of reverse transcriptase (RT) activity released into the supernatant, as described (12). The detection of virion-associated RT activity in culture supernatants is a surrogate marker for *de novo* virus production.

3.1.6 *Quantification of HIV-1 DNA by real-time PCR.* Unpolarized control, M1- and M2a-MDM were infected with DNAse/RNAse-free HIV-1_{BaL} (m.o.i.=0.1) and were then cultivated in complete medium for 48 h, a period estimated to be required for completion of a single round of HIV replication in macrophages (31). The infected MDM (10^6 cells/mL) were then washed, resuspended in lysis buffer containing polyoxyethylene 10 lauryl ether (0.1%) and proteinase K (0.1 mg/mL) from Sigma and digested at 65 °C for 2 h; proteinase K was then heat-inactivated at 95 °C for 15 minutes (32). An amount of lysate corresponding to $2.5x10^4$ cells was amplified by real-time quantitative PCR reactions using primers and a probe that recognize the HIV-1 *gag* gene (33, 34): forward primer 5'-ACATCAAGCAGCCATGCAAAT-3'; reverse primer 5'-ATCTGGCCTGGTGCAATAGG-3'; probe 5'-(FAM) CATCAATGAGGAAGCTGCA GGAATGGGATAGA (TAMRA)-3'. This primer/probe combination detects all forms of viral DNA synthesized after second-strand transfer mediated by RT. The number of HIV-1 DNA copies were normalized to those of human GAPDH by an external standard curve showing a linear distribution (r=0.99) between 10 and 10^6 copies[40]. The primers and probe for GAPDH were: forward primer 5'-ACCACAGTCCATGCATCACT-3'; reverse primer 5'-GGCCATCACGCCA CAGITT-3'; probe 5'-(FAM) CCCAGAAGACTGTGGATGGCCCC (TAMRA)-3'. Thermal cycling conditions for real-

time PCR with SYBR Green quantification were 50 °C for 2 min and 95 °C for 15 min followed by 40 cycles at 95 °C for 15 sec and 65 °C for 1 min (34).

3.1.7 *Western blot analysis of cell-associated HIV-1 proteins.* Cells were scraped from the wells with a rubber policeman and lysed with 100 ul of buffer C per million cells (20 mM Hepes, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM $MgCl_2$, 0.5% glycerol). Cellular proteins were denatured in an equal volume of 2x sample buffer (0.125 M Tris-HCl, pH 6, 8; 4% SDS; 10% 2–2-ME; 20% glycerol) for 5 min at 100°C, separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Hybond ECL; Amersham) by electroblotting. Membranes were blocked in 5% nonfat milk, 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.2% Tween 20 for 30 minutes at room temperature and further incubated for 1 hour with the serum (1:1000 dilution) from an HIV-1-infected individual containing high titers of anti-HIV-1 Abs. Ab binding was visualized by using HRP-conjugated anti-human Abs (Amersham Pharmacia Biotech, Piscataway, NJ). The signal was detected using ECL reagents (Amersham) according to manufacturer's instructions.

3.1.8 *Statistical analysis.* Prism 5 from GraphPad Software (La Jolla, CA) was used for statistical analyses. Results are reported as mean values $\pm$ SD. To minimize inter-donor variability, values were normalized relative to unstimulated control cells. One-way ANOVA and the Tukey post-test were used for multivariate analysis. To further compensate for inter-donor variability, all assays were performed on triplicate samples with MDM derived from 4 to 6 independent donors, as further specified.

3.2 RESULTS

3.2.1 *Both M1 and M2a polarization of human MDM results in the inhibition of HIV-1 replication.* To assess the relative capacities of M1- and M2a-MDM to support productive infection, MDM were established from 15 independent HIV-1 seronegative donors and were either left unpolarized (controls) or were polarized with M1 (TNF-α plus IFN-γ) or M2 (IL-4) inducing cytokines for 18 h, as previously described (Chapter 2), prior to infection with R5 HIV-1_{BaL}. Virus replication in control MDM increased progressively reaching peak levels 14-18 days post-infection followed by a drop to low levels of persistent replication (**Figure 3.1 A**). In contrast, HIV-1 replication was strongly inhibited in polarized MDM with M1 cells showing a significantly stronger inhibition in comparison to M2a-MDM (the mean inhibition of peak RT activity levels in M1- and M2a-MDM vs. control cultures was 92% and 62%, respectively). Although M2a polarization decreased replication in all cell cultures, it did not affect the kinetics of HIV spreading as measured by peak RT activity (**Figure 3.1 A**). The levels of viral suppression in M1-MDM ranged from 51-75% in 20% of independent cultures to 76%-100% in the remaining 80% of cultures (**Figure 3.1 B**). In comparison, the levels of suppression in M2a macrophages ranged from <25% in 7% to 26-50%, 51-75% and >76% in 7%, 33% and 53% of independent MDM cultures, respectively (**Figure 3.1 B**).

3.2.2 *M1 and M2a polarization differentially modulates the expression of receptors and chemokines involved in HIV-1 entry.* In order to investigate the effects of M1 and M2a polarization on MDM susceptibility to HIV-1 infection, I monitored changes in the expression of CD4 and CCR5 the primary receptor and the major co-receptors for HIV-1 entry into monocytes and macrophages, respectively (35, 36). M1, and to a lesser extent M2a polarization resulted in a significant reduction in the percentage of MDM expressing CD4, a finding that may account, at least in part, for their decreased capacity to support productive infection (**Table 1**). In contrast, expression of CCR5, the major co-receptor for

macrophage-tropic strains of HIV-1, remained unchanged and, in some donors, was upregulated on the surface of M2a-MDM vs. M1- and controls (**Table 1**).

Next, I investigated the effects of M1 and M2a polarization on the secretion of the CCR5-binding chemokines CCL3, CCL4 and CCL5. These chemokines are well-characterized potent inhibitors of R5 HIV-1 entry in $CD4^+$ T lymphocytes as well as in MDM (16, 37). Secretion of CCL3 and CCL4 was detected in all donors and was significantly upregulated in M1- vs. M2-MDM (25-fold in M1 vs. approximately 2-fold in M2-MDM for CCL3; 14-fold in M1 vs. <2-fold in M2 for CCL4) (**Figure 3.2 A**). CCL5, which was undetectable in both control and M2-MDM, was significantly upregulated in M1-MDM (range: 61-186 pg/mL) from 3 of 6 donors (**Figure 3.2 A**). The increased production in CCL3, CCL4 and CCL5 (in some donors) suggests that these chemokines may contribute to the polarization-induced inhibition of HIV-1 replication in M1-MDM by preventing or curtailing virus entry.

3.2.3 *M1, but not M2a, polarization restricts HIV-1 entry into MDM.* To further examine the mechanisms underlying the inhibition of HIV-1 replication in M1- vs M2a-MDM I quantified the amount of viral DNA that accumulated during the first 48 h of infection, a time considered sufficient for a single round of virus replication in these cells (31). M1 polarization consistently led to a marked decrease in the accumulation of HIV-1 DNA (range: 54-97%) by 48 h after infection. In contrast, no statistically significant differences were observed in the amount of HIV-1 DNA that accumulated in M2a- and control MDM at this time point (**Figure 3.2 B**), indicating a clear-cut difference in the HIV-inhibitory mechanisms induced by M1 vs. M2a polarization.

3.2.4 *M2a polarization restricts HIV-1 production at the level of viral assembly and/or budding.* To further delineate the mechanisms underlying the inhibition of HIV-1 infection in M1- versus M2a-MDM, I measured the accumulation of viral proteins during the first 7 days of infection using western blot methodology. As expected, M1 polarization was associated with a marked decrease in the accumulation of HIV-1 proteins relative to

control cells (**Figure 3.3 B**). This data is consistent with the low levels of viral DNA in these cells. In contrast, M2 polarization had no effect on the synthesis or accumulation of viral proteins despite the marked reduction in the production and release of HIV-1 virions into the culture supernatant (**Figure 3.3 A**). The finding that HIV-1 protein levels in M2a-MDM were similar to those detected in control MDM (**Figure 3.3 B**) provides further confirmation that the restriction of HIV-1 infection M2a occurs at a late step in the viral life cycle (ie. post-transcription and post-translation).

3.2.5 *Polarization-induced inhibition of virus replication is transient and of shorter duration in M1- vs. M2a-MDM.* Next, I examined the duration of the anti-HIV-1 responses following a single 18 h exposure to M1 and M2a-inducing cytokines. For these studies, control and polarized MDM were thoroughly washed and infected with HIV-1_{BaL} either immediately (day 0) or on days 3 and 7 post-polarization. Exposure to HIV-1 immediately after polarization led to a rapid decrease in HIV-1 replication in both M1- and M2a-MDM relative to control cells. However, the kinetics and duration of the inhibitory response differed in M1 and M2-MDM with the nadir of inhibition in M2a-MDM occurring on day 10 (**Figure 3.4, upper left panel**). A more prolonged suppression of virus replication lasting for up to 25 days was observed in M1-MDM (**Figure 3.4, upper left panel**). When infection was delayed until day 3 post-polarization, M2a-MDM still maintained their ability to inhibit HIV-1 replication, whereas M1-MDM had already lost their inhibitory phenotype in that they supported HIV replication at the same levels of control cells (**Figure 3.4, upper right panel**). When M1- and M2a-MDM were infected 7 days after polarization, replication levels were similar to those observed in control cells (**Figure 3.4, lower panel**). Thus, the inhibitory effects of MDM polarization, particularly in the case of M1-MDM, are transient and do not render macrophages permanently refractive to HIV-1 infection and replication.

In parallel to these virological findings, removal of the polarizing stimuli led to a near complete reversal of M1 and M2a cytokine/chemokine profiles (Chapter 2) and cell

surface phenotypes by day 7. Significantly, three days after polarization, cell surface CD4 expression had returned to control levels in M1-MDM, whereas the return of CD4 expression on the surface of M2a-MDM to control levels occurred more slowly (**Table 2**).

3.3 DISCUSSION

In this chapter, I demonstrate that MDM polarization into either M1 or M2a macrophages leads to a restriction in their capacity to support productive HIV-1 infection. Furthermore, I demonstrated that such restrictions occur at different levels of the viral life cycle (i.e. entry/early, pre-integration events for M1- and post-transcription and translation for M2a-MDM). Macrophage polarization into an M1 phenotype was associated with a more profound suppression of HIV-1 replication and a more dramatic modulation of CD4 and β-chemokines compared to M2a-MDM. The downregulation of CD4 and increased secretion of CCL3, CCL4 and CCL5 correlated with a sharp decrease in HIV-1 DNA synthesis at 48 h, a decrease in the synthesis and accumulation of HIV-1 proteins at day 7 and a loss of inhibition in M1 cells infected 3 days after polarization. In contrast, M2a polarization was associated with a less profound but more sustained inhibition of virus replication with no detectable impairment of virus entry/reverse transcription or protein accumulation. Finally, I demonstrate that the transient and reversible nature of M1- and M2a-MDM polarization (for most determinants, see Chapter 2) is associated with the loss HIV-1 inhibition.

Collectively, my results indicate that impairment of early events in the HIV life cycle (from viral entry to reverse transcription) is the main, if not sole, mechanism responsible for inhibition of HIV-1 replication in M1-MDM. This interpretation is consistent with previous reports showing that both IFN-γ and TNF-α can downregulate CD4, thereby preventing or limiting HIV-1 entry. In this regard, previous studies have shown that IFN-γ decreased CD4 expression in monocytes, while TNF-α, either alone or in combination with IL-13 downregulated CD4, as well as CCR5 and CXCR4, from the surface of MDM (16). My study confirms and extends these observations to M1-MDM generated by the short-term co-stimulation of MDM with TNF-α plus IFN-γ. Interestingly, however, I found no decrease in CCR5 levels in spite of the high-level secretion of CCL3, CCL4 and CCL5, the natural ligands of CCR5. These some what counter intuitive results

are likely explained by the transient nature of the polarization phenotype induced by short term stimulation.

While the down regulation of CD4 and up-regulation of beta-chemokines explains the inhibitory phenotype of M1 MDM, the severity of inhibition makes it tempting to speculate that additional factors may contribute to the phenotype. Consistent with early/preintegration inhibition of M1 MDM, APOBEC3G expression increases in MDM stimulated with IFN-α, but also, although to a lesser extent, with IFN-γ (38). A cellular protein, APOBEC3G is incorporated into HIV-1 virions and exerts its antiviral activity after the virus has entered a new target cell by introducing C-to-U changes in the newly synthesized single stranded cDNA (39). This, in turn, leads to G-to-A hypermutations in the sense strand of retroviral DNA, a process that can lead to lethal mutagenesis in a single replication cycle. Although not shown here, preliminary data clearly show that APOBEC3G is up-regulated in our M1 MDM model system.

Another salient feature of M1 polarization of MDM, as discussed in Chapter 2, was the strong and sustained upregulation of CXCL10 (at least up to 7 days post-polarization), a chemokine that plays an important role in promoting HIV-1 encephalitis (40, 41). Previous studies have shown that CXCL10 stimulates HIV replication by downregulating the secretion of CCR5-binding chemokines, thereby enhancing viral entry (42-44). CXCL10 associated enhancement of HIV-1 infection was not observed in our system, likely as a result of the early potent inhibitory effects of TNF-α and IFN-γ on CD4 expression, the upregulation of CCR5-binding chemokines and the resultant decrease in HIV-1 DNA accumulation. However, the prolonged secretion of CXCL10 following M1 polarization may have contributed to the downregulation of beta-chemokine secretion in these cells (see Chapter 2).

CD4 was also downregulated in M2a-MDM, but to a lesser extent than in M1-MDM (**Table 1**). As previously reported for monocytes preincubated with IL-4 for 5 days (45), however, the decrease in CD4 on M2a-MDM did not lead to a reduction in viral entry

or in the accumulation of HIV-1 DNA at 48h and cannot explain the inhibitory phenotype of M2a MDM. Interestingly, in monocytes exposed to IL-4 before infection, virus production is highly dependent on an inhibition of cell proliferation during *in vitro* differentiation into macrophages (45). In my studies, the IL-4-dependent increase in CCR5 expression observed in some donors, (**Table 1**) may have compensated for the decreased levels of CD4 expression on M2a cells. This interpretation is consistent with the observation that macrophages expressing high levels of CCR5 and low levels of CD4 are fully susceptible to infection by R5 HIV-1, such as HIV-1_{BaL} (46). A feature that could potentially account for the post-entry restriction of HIV expression in our M2a-MDM is the accumulation of transcriptional inhibitory p50 homodimers in the cell nucleus (47-49). While we investigated this hypothesis, we did not find evidence of such a phenomenon, at least not under the experimental conditions used in our study (data not shown).

The finding that the synthesis and accumulation of HIV-1 proteins was unaffected by M2a polarization suggests that the block in HIV-1 replication in M2a-MDM occurs at a post-transcription/post-translation step of the viral life cycle, most likely at the level or virion assembly or budding. This is in contrast to a study published by Schuitemaker et al. who reported that the IL-4-induced inhibition of HIV-1 replication in MDM occurs at the level of reverse transcription (45). This apparent discrepancy is most likely due to differences between the two MDM models. Unlike our 18-hour polarization protocol, they differentiated monocytes for 5 days in the presence of IL-4 prior to infection (45). Other studies have shown that exposure of MDM to IL-10 prior to infection inhibits HIV-1 replication at the level of protein processing suggesting that M2a and M2c may inhibit HIV at similar levels (50).

Another distinctive feature of M2a-MDM from Chapter 2 that may have relevance on HIV replication, was the persistent upregulation of CCL22 secretion. This was not observed in either control or M1-MDM. In this regard, CCL22 was originally described as a suppressive factor released by activated $CD8^+$ T cells as part of their repertoire of non-

lytic soluble inhibitory factors of HIV (51). Although this observation was not confirmed by all laboratories (52, 53), we have reported that CCL22 inhibits HIV-1 in MDM but not in activated PBMC by acting at a post-entry step of the viral life cycle (12). This data is consistent with the inhibition of HIV-1 replication observed with our M2a-MDM.

CCL2, a pro-inflammatory chemokine that was constitutively secreted by M1 and control MDM, was downregulated in M2a-MDM (see Chapter 2). In this regard, CCL2 is involved in the early recruitment of monocytes to inflammatory lesions (14) and has been shown to enhance the replication of X4 HIV-1 strains in activated PBMC (54) whereas neutralization of this chemokine has been shown to inhibit the late phase of virion release in primary MDM (55). Thus, the reduction in CCL2 secretion together with increased CCL22 production may have contributed to the prolonged post-transcription/translation inhibition of HIV-1 replication observed in M2a-, but not in M1-, MDM.

Both M1- and M2a phenotypes were reversible 3 to 7 days after removal of the inductive stimuli, although with some exceptions. Of interest, I observed symmetrical contra-modulatory effects temporally associated with the loss of M1 and M2a polarization. This phenomenon may amplify and prolong the consequences of individual polarization programs. In this regard, a strong correlation was observed between the return to a non-polarized state and the loss of inhibition of viral replication, with M1 cells exhibiting an earlier recovery in both the expression of surface membrane CD4 and the capacity to support HIV replication compared to M2a-MDM. With the exception of CXCL10 (M1) and CCL22 (M2a), both M1- and M2a-MDM showed a complete reversion to control levels 7 days after the removal of polarizing cytokines. The transient nature of M1 and M2a phenotypes, at least for most determinants, suggests that the observed restrictions in virus replication are linked to the complex and multi-factorial polarized phenotypes.

In conclusion, my findings suggest that cytokine-mediated polarization of mature macrophages, as investigated in human MDM, may be an important regulator of macrophage susceptibility to HIV-1 infection and/or replication in polarized M1 and M2a

cells. The transient and reversible nature of the dynamic changes in cell phenotype and activation status may represent a mechanism through which these cells, that are typically resistant to the cytopathic effect of HIV-1(3-5), cycle between a state of resistance or latent infection and productive viral expression and spreading.

3.4 TABLES

Table1. Fold changes of HIV receptor and co-receptor expression in M1- and M2a- vs. Control MDM.

Receptor	N°	Parameter	Average Expression (control)	M1 (fold over vs. Cont)	M2a (fold over vs. Cont)	M1 vs. Cont	M2a vs. Cont	M1 vs. M2a
CD4	5	Positive cells	41%±3.3	0.14±0.08	0.32±0.08	**	**	*
		MFI	12±2.3	0.92±0.07	0.91±0.05	*	*	
CCR5	5	Positive cells	21%±1.8	1.27±0.06	1.87±0.72			
		MFI	44±3.1	0.98±0.01	0.91±0.11			

Mean fold change in the number of positive cells and MFI in M1- and M2a-MDM vs. control MDM. MFI and expression of surface receptors/co-receptors was analyzed by cytofluorimetry. *$p<0.05$,**$p<0.001$ as assessed by one way ANOVA and Tukey post-tests. N° denotes the number of MDM donors studied.

Table 2. Kinetics of CD4 expression following MDM polarization.

Days after polarization	N°	Polarization	% $CD4^+$ MDM	M1 vs. Control	M2a vs. Control	M1 vs. M2a
0	5	Control	41±3.3	**	**	*
		M1	6±1.9			
		M2a	13±2.6			
3	5	Control	35±6.7		*	*
		M1	31±9.2			
		M2a	13±5.5			
7	5	Control	33±4.8			
		M1	31±6.9			
		M2a	26±2.9			

Mean CD4 expression on control, M1- and M2a-MDM. *$p \leq 0.05$,**$p \leq 0.001$ as assessed by one way ANOVA and Tukey post-test. N° denotes the number of individual MDM donors tested.

3.5 FIGURE LEGENDS

Figure 3.1 Stronger inhibition of R5 HIV-1 replication in M1- vs. M2a-MDM. **A.** The effects of MDM polarization on HIV-1 replication kinetics were monitored by determination of the RT activity content in culture supernatants over a 35-day infection period. The results represent the average replication (±SD) of infected MDM cultures established from 15 independent donors. **B.** Pie chart showing the percent levels of suppression of HIV-1 in the 15 infected MDM cultures studied. Values ranged from near-complete/complete (76%-100%) inhibition in 80% of independent M1-MDM cultures to 51%-75% in the remaining 20% of cultures. Lower levels of inhibition of virus replication were observed in M2a-MDM.

Figure 3.2 Differential secretion of CCR5-binding chemokines in M1- and M2a-MDM is associated altered accumulation of HIV-1 DNA. A. Levels of CCL3, CCL4 and CCL5 secretion in control, M1- and M2a-MDM determined after 18 h of cultivation in the presence of polarizing cytokines, expressed as pg/mL. These results represent the mean (±SD) levels of cytokines secreted by MDM cultures established from 6 independent donors. **B.** Control, M1- and M2a-MDM were infected with HIV-1_{BaL} and harvested after 48 h. Levels of HIV-1 *gag* DNA ranged from 52 to 4,463 copies per 1,000,000 cells in infected MDM, while uninfected MDM routinely expressed <1 copy viral RNA per 1,000,000 cells. These results represent the mean (±SD) of 8 individual infections of independent MDM cell cultures. Statistical differences were assessed using the multivariate one-way ANOVA and Tukey's post-test.

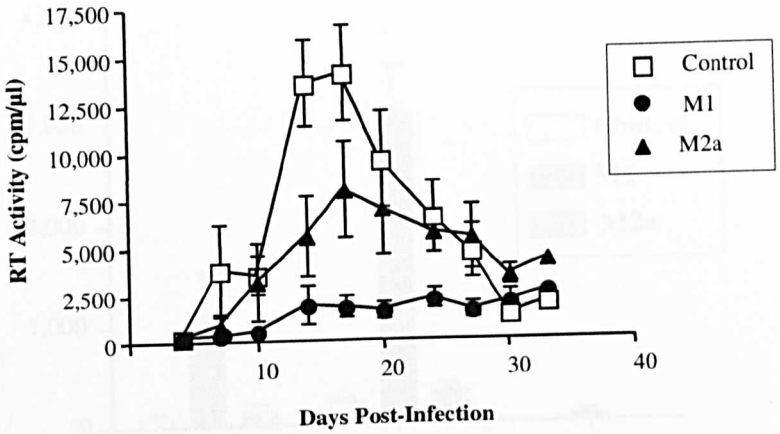
Figure 3.3 M1 but not M2a polarization is associated with decreased viral protein accumulation. A. Control, M1 and M2a were infected with HIV_{BAL} (m.o.i. 0.1) and RT activity was assessed in culture supernatant 7 days post-infection. The same cells were also used in Western blot analysis. **B.** Accumulation of viral proteins was also determined in control, M1- and M2a-MDM 7 days post-infection using the serum from an

HIV-infected individual with high anti-HIV antibody titers. Results are representative of 3 independent experiments.

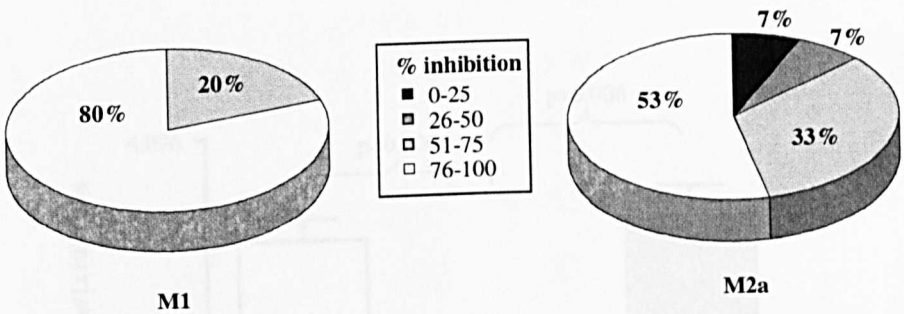
Figure 3.4 Time-dependent kinetics of inhibition of HIV-1 replication in M1- and M2a-MDM. M1-, M2a- or control MDM were infected either immediately after polarization (day 0) or on days 3 and 7 after polarization, as indicated by the arrows. The results are shown as mean fold change in RT activity relative to unpolarized control MDM. These results were obtained from a single MDM culture representative of 5 cultures established from independent donors. The inhibitory effects were completely lost when M1-MDM were infected 3 days after polarization, unlike what was observed for M2a-MDM. The apparent increase in the replicative capacity of M1- and M2a-MDM observed at late time points in cells infected on days 0 or 7 is explained by the low levels of virus replication detected in control MDM cultures.

Figure 3.1

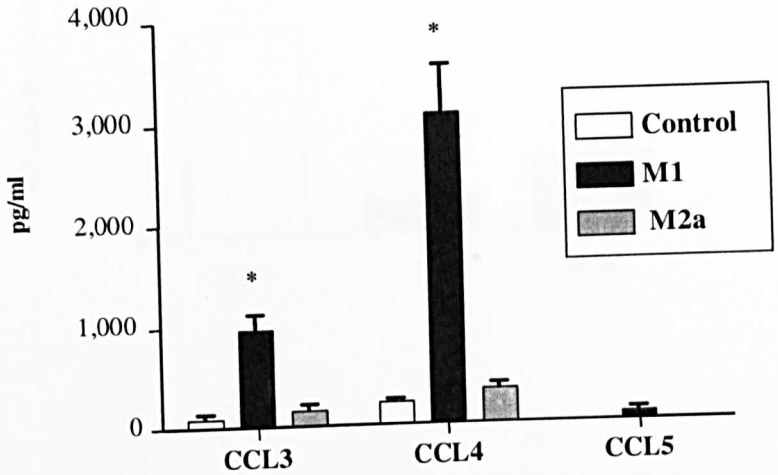
A.


17,500
15,000
12,500
10,000
7,500
5,000
2,500
0
RT Activity (cpm/µl)
10
20
30
40
Days Post-Infection
Control
M1
M2a

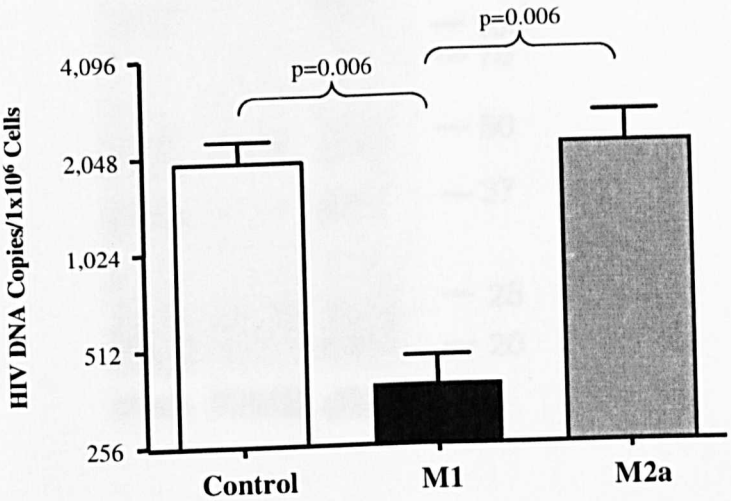
B.


80%
20%
M1
% inhibition
0-25
26-50
51-75
76-100
7%
7%
53%
33%
M2a

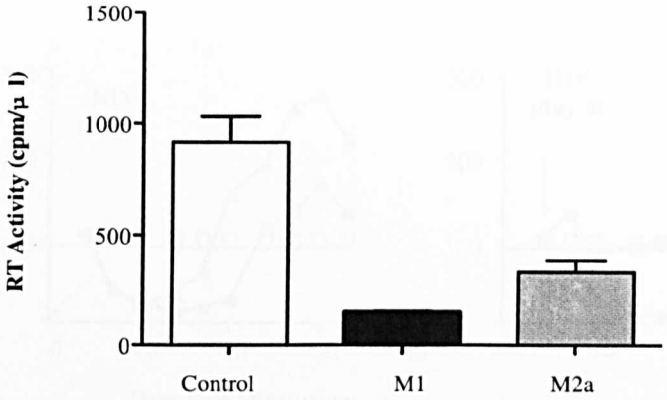
A.


4,000
3,000
2,000
1,000
0
pg/ml
*
*
Control
M1
M2a
CCL3
CCL4
CCL5

B.


p=0.006
p=0.006
4,096
2,048
1,024
512
256
HIV DNA Copies/1x10⁶ Cells
Control
M1
M2a

A.


1500
1000
500
0
RT Activity (cpm/µ l)
Control
M1
M2a

B.

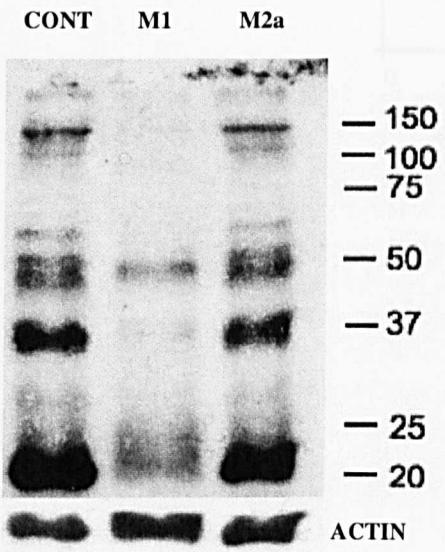
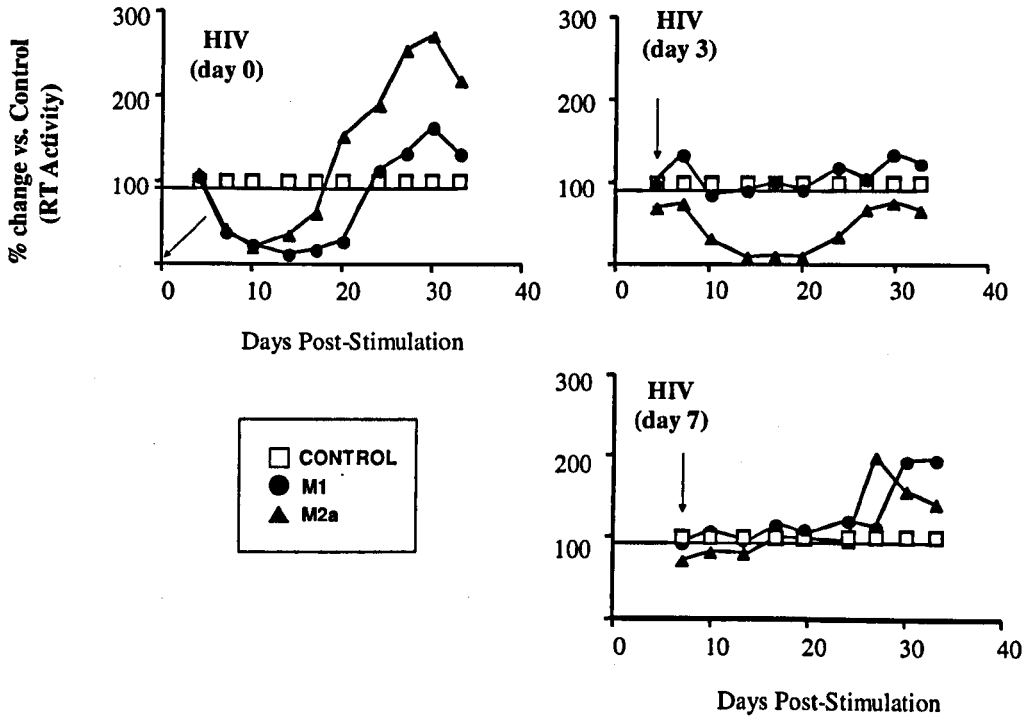

CONT
M1
M2a
150
100
75
50
37
25
20
ACTIN

Figure 3.4


% change vs. Control (RT Activity)
300
200
100
HIV (day 0)
HIV (day 3)
HIV (day 7)
0
10
20
30
40
Days Post-Stimulation
CONTROL
M1
M2a

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CHAPTER 4
RESTRICTED PRODUCTION OF HIV-1 IN DC-SIGN+ M2a MDM IS ASSOCIATED WITH EFFICIENT TRANSMISSION OF HIV-1 TO PBMC

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4.0 INTRODUCTION

Cells of the macrophage lineage are one of the first target cells infected by HIV-1 and they play a critical role in the cell-to-cell spread and dissemination of the virus, particularly in the central nervous system (CNS) (1-3). Macrophages are relatively resistant to the cytopathic effects of HIV-1 and because of their longevity, abundance and ubiquitous distribution in peripheral tissues serve as long-lived reservoirs of viral infection (4-6). However, the ability of macrophages to support productive HIV-1 infection is profoundly influenced by cytokines and chemokines in the microenvironment (7-11). In Chapters 2 and 3, I demonstrated that macrophages can be polarized into M1 (pro-inflammatory) or M2a (anti-inflammatory) populations that differ in their phenotypic, functional and regulatory properties, and in their ability to support virus production (12-14). Another important finding arising from this work, with particular importance for HIV pathogenesis, was the finding that DC-SIGN was differentially regulated on M1 (down-regulation) versus M2a- (upregulation) MDM. Since DC-SIGN mediates the capture and transmission of HIV-1 from dendritic cells (DC) to CD4 T cells *in vitro* (15,16), it has been proposed that this molecule may play an important role in the dissemination and transport of HIV-1 from sites of mucosal transmission to T cell areas of lymphoid tissues.

Numerous studies have reported that the DC-mediated transmission of HIV-1 involves the capture of extracellular virions by DC-SIGN and the subsequent transfer of this virus to permissive T cells, with or without internalization (15-18). In contrast, the mechanisms underlying the transmission of HIV-1 from macrophages to T cells have not been thoroughly investigated. Similar to DC, MDM-mediated transmission appears to take place across a transient virological synapse (VS) formed at the MDM-T cell interface (19). Assembly of the VS is driven by the co-polarization of HIV-1 Gag and Env in the MDM

with CD4+ on T cells and is most likely stabilized by cell-cell adhesion interactions (19). Other studies have shown that in HIV-1-infected macrophages accumulate virions in multivesicular bodies (MVB) of the exocytic pathway and in complex invaginated plasma membrane domains (19-21). Virions sequestered in these compartments remain infectious for at least six week and during VS formation rapidly move to the site of cell-cell contact for presentation to susceptible T cells (22).

In this Chapter, I investigated the effects of M1 and M2a polarization on the HIV-1 transmission properties of MDM. As discussed in Chapter 2, DC-SIGN was strongly and differentially upregulated on the surface of M2a MDM and remained above control levels up to 7 days post-stimulation. Despite a significant downregulation of CD4 receptors on M2a MDM (Chapter 2 and 3), $CD4^{low+}$/DC-$SIGN^{high+}$ M2a and $CD4^{high+}$/DC-$SIGN^{low+}$ control MDM bound similar levels of virus. However, only M2a-MDM were capable of efficiently transmitting HIV-1 to susceptible target cells. In addition to binding, DC-SIGN also compensated for the loss of CD4 receptors on M2a-MDM by enhancing viral entry and reverse transcription. Finally I found that the sustained upregulation of DC-SIGN on M2a cells was associated with a decreased capacity to support productive HIV-1 infection, possibly through DC-SIGN- mediated signaling events. Collectively, these data suggest that DC-SIGN expression on M2a macrophages play an important role in the regulation of *cis*- vs. *trans*-infection. By enhancing HIV-1 entry and inhibiting viral replication, DC-SIGN may also contribute to the development of latent infection in macrophages of the M2a phenotype.

4.1 MATERIALS AND METHODS

4.1.1 *Reagents.* Human recombinant cytokines were purchased from R&D Systems (Minneapolis, MN) and used at concentrations of either 2.0 (TNF-α) or 20.0 (IFN-γ, IL-4) ng/mL. All cytokines were declared by the manufacturer to contain <0.1 ng of contaminating LPS per μg of protein. Ficoll-Hypaque was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). D-MEM, PBS, Fetal Bovine Serum (FBS), Normal Human Serum (NHS), penicillin, streptomycin and glutamine were obtained from Cambrex (Verviers, Belgium). Anti-CD4 (Leu3a) and anti-DC-SIGN (clone 612) antibodies were purchased from Becton Dickenson (Frankline Lakes, New Jersey) and R&D Systems respectively. The RETROtek P24 Antigen ELISA assay was obtained from ZeptoMetrix (Buffalo, New York).

4.1.2 *Isolation of human monocytes and differentiation into monocyte-derived-macrophages (MDM).* Peripheral blood mononuclear cells (PBMC) were isolated from the buffy coats of healthy HIV-1-seronegative blood donors by Ficoll-Hypaque density gradient centrifugation. The cells were washed, resuspended in D-MEM containing 1% pen/strep, 1% glutamine, 10% heat-inactivated FBS and 5% heat-inactivated NHS (complete medium) and seeded into 75 cm^2 flasks (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) at a concentration of $8x10^6$ cells/mL. After 2 h of incubation at 37° C in a humidified atmosphere containing 5% CO_2, the non-adherent cells including T lymphocytes were removed by gentle pipette aspiration. An equivalent volume of fresh complete medium was then added to each flask. Adherent cells were cultured for an additional 24 h, washed twice with PBS and detached from the flask by scraping with a rubber policeman. Following re-suspension, the cells were tested for viability by Trypan-blue exclusion and counted by haemocytometer. The purity of these monocyte isolations, as determined by anti-CD14-PE staining and flow cytometry, exceeded 85%. The cells were then seeded into 48-well plastic plates (Falcon) at the concentration of $2.5x10^5$ cells/well and cultivated for 7-8 additional days at 37 °C in 5% CO_2 to promote their full

differentiation into MDM(7). MDM (≥95% $CD14^+$) were stimulated for 18h with either IL-4 (M2a) or TNF-α plus IFN-γ (M1) cytokines (23). Following 18 h of cytokine stimulation, M1-, M2a- and unstimulated (control) MDM were washed with cytokine-free medium and analyzed in parallel. To investigate the sustainability of the polarizing response, M1-, M2a- and control MDM were thoroughly washed and cultivated for 7 additional days in absence of polarizing cytokines. Changes in the expression of DC-SIGN were measured at days 0, 3 and 7 post-polarization.

4.1.3 *Flow cytometric analysis of DC-SIGN and CCL3 expression.* Adherent MDM were washed with cold EDTA/PBS (2 mM) and detached from the plastic plates by incubation with cold EDTA/PBS (2 mM) for 30 min at 4 °C and scraping with a rubber policeman. Non-specific binding of Ab was blocked by pre-incubating the cells in medium containing 15% serum (10% FBS and 5% NHS) for 15 min at 4 °C. Cells were incubated with anti-DC-SIGN (PE) from R&D Systems for 30 minutes at 4°C, washed with cold PBS and fixed with 2% paraformaldehyde (PFA). Acquisition of flow cytometric data was performed using a CYAN ADP cytometer (DAKO Cytomation, Glostrup, Denmark) and the results were analyzed with the FlowJo software version 8.4.3 (Tree Star Inc., Ashland, OR).

Intracellular staining for CCL3 expression was determined after 18 h of polarizing cytokine stimulation in medium containing BFA (1 μg/ml). Following cytokine stimulation, M1- and M2a-MDM were detached from the plastic surface by scraping and fixed in 2% PFA. The cells were labeled with anti-DC-SIGN Ab (as described above), permeabilized with Saponin buffer (0.1% Saponin, 0.5% BSA in PBS) and stained with Allophycocyanin conjugated anti-CCL3 Ab for 30 min at 4°C. After staining, the cells were washed twice with cold PBS and analyzed with a CYAN ADP apparatus (DAKO Cytomation).

4.1.4 *Microscopic evaluation of DC-SIGN expression on MDM.* Control, M1 and M2a MDM were washed twice with cold DMEM containing 10 % FCS and 5% NHS and

stained with 25 µl/well of PE-conjugated anti-DC-SIGN mAb (R&D Systems) for 30 min at 4 °C. Cells were then washed 4 times with cold PBS and fixed with 4% PFA in PBS. Nuclei were stained by adding 1 µg/ml of Hoechst 33258, pentahydrate (bis-benzimide, m.w. of 623.96, Molecular Probes) for 15 min at RT. After two more washes in PBS, cells were covered with 200 µl of PBS and analyzed by IN Cell analyzer 1000 (Amersham). Each condition was assayed in triplicate (for a total of 800-1200 cells per condition, image magnification 20x). DC-SIGN+ cells were recognized when fluorescence intensity was greater than 50% that of background.

4.1.5 *HIV-1 binding assay.* Control, M1- and M2a-MDM were pulsed with the macrophage-tropic, CCR5-dependent (R5) reference strain HIV-1_{BaL} at a multiplicity of infection (m.o.i.) of 1.0 for 2 hours at 4°C. The cells were then washed thoroughly (to remove unbound virus), lysed with 50 µl of lysis buffer and processed for HIV-1 Gag quantification using the RETROtek P24 antigen ELISA assay as specified in the manufacturer's instructions. For antibody blocking experiments, polarized and control MDM were pre-incubated with 20 µg/ml of anti-CD4 or anti-DC-SIGN Ab, either alone or in combination, for a period of 30 min at room temperature.

4.1.6 *Quantification of HIV-1 DNA by real-time PCR.* Control, M1- and M2a-MDM were infected with DNAse/RNAse-free HIV-1_{BaL} at m.o.i. of 0.1. In antibody-blocking experiments, the MDM were pretreated 20ug/ml anti-DC-SIGN mAb (R&D Systems) for 30 minutes at room temperature prior to infection. Following infection, the MDM were cultivated in complete medium for 48 h, the estimated time required to complete a single viral replication cycle in macrophages (24). The infected MDM (10^6 cells/mL) were then washed, resuspended in lysis buffer containing 0.1% polyoxyethylene 10 lauryl ether (Sigma) and 0.1 mg/mL proteinase K and digested at 65 °C for 2h; the proteinase K was then heat-inactivated at 95 °C for 15 minutes (25). For each PCR reaction, an amount of lysate corresponding to 2.5 x 10^4 cells was amplified in real-time

quantitative PCR reactions using primers and a probe that recognize the HIV-1 *gag* gene (26, 27): forward primer 5'-ACATCAAGCAGCCATGCAAAT-3'; reverse primer 5'-ATCTGGCCTGGT GCAATAGG-3'; probe 5'-(FAM) CATCAATGAGGAAGCTGCAGGAATGGGATA GA (TAMRA)-3'. This primer/probe combination detects all viral DNA that is synthesized after second-strand transfer including both pre- and post-integration DNA. HIV-1 copy numbers were normalized against human GAPDH as determined using an external standard curve with a linear distribution ($r = 0.99$) between 10 and 10^6 copies. The primers and probe for GAPDH were: forward primer 5'-ACCACAGTCCATGC ATCACT-3'; reverse primer 5'-GGCCATCACGCCACAGITT-3'; probe 5'-(FAM) CC CAGAAGACTGTGGATGGCCCC (TAMRA)-3'. Thermal cycling conditions for real-time PCR with SYBR Green quantification were: 50 °C for 2 min and 95 °C for 15 min followed by 40 cycles at 95 °C for 15 sec and 65 °C for 1 min (27).

4.1.7 *HIV-1 infection and replication.* Separate control, M1- and M2-MDM cultures were incubated for 20 minutes at room temperature in the presence (or absence) of anti-CD4 or anti-DC-SIGN blocking antibodies, prior to infection with HIV-1_{BaL} at an m.o.i. of 0.1. To assess virus production and spread, replicate aliquots of the culture supernatant were collected every 3 days over a 12-day period and stored at -20° C. At the end of each culture period, the supernatants were thawed and analyzed for viral replication as determined by measuring the amount of HIV-1 reverse transcriptase (RT) activity released into the supernatant, as previously described (7)

4.1.8 *HIV-1 transmission assay.* Polarized and control MDM pre-treated with and without anti-DC-SIGN mAb (20 µg/ml; 20 min at room temperature) were incubated with HIV_{IIIB} (X4, m.o.i. 0.1) for 2 hours at 37°C. These control, M1- and M2-MDM were then washed extensively to remove unbound virus and co-cultured (1:1 ratio) with monocyte-depleted IL-2-treated PBMC for either 6 hours or 12 days. The separated PBMC (removed after 6 hours) were then washed, re-plated in fresh media and maintained in culture for an

additional 12 days. Supernatants from PBMC and MDM-PBMC cultures were collected at regular intervals (every 3 days) over a 12-day period and stored at -20° until analyzed. Ongoing viral replication was determined by assessing RT activity in culture supernatants as previously described (Chapter 3).

Statistical analysis. Prism 5 from GraphPad Software (La Jolla, CA) was used for statistical analyses. Results are reported as mean values $\pm$ SD. Multivariate analyses were conducted using one-way ANOVA and the Tukey post-test. To compensate for inter-donor variability, all assays were performed on triplicate samples with MDM derived from 4 to 8 independent donors, as further specified.

4.2 RESULTS

4.2.1 *DC-SIGN is upregulated on M2a-, but not M1-MDM.* Of particular interest in clarifying the potential role of macrophages in the cell-mediated spread of HIV-1, was the finding that DC-SIGN was the only receptor to show a divergent pattern of expression on control, M1- and M2a-MDM. This receptor, which was expressed at relatively low levels on control MDM, was strongly upregulated on the surface of M2a-MDM both in terms of the percentage of positive cells (9-fold increase) and MFI (2-fold increase) (**Figure 4.1 A and** Chapter 2). This is in clear contrast to the near-complete down-regulation of DC-SIGN on the M1 cells of most donors (**Figure 4.1 A and** Chapter 2). Microscopic examination of MDM stained with PE-labeled anti-DC-SIGN mAb confirmed the results obtained by flow cytometry. On average, 50.7% of M2a, 10.2% of control and only 4.6% of M1-MDM tested positive for DC-SIGN (**Figure 4.1 B**).

To establish whether a single 18 hour exposure to IL-4 could induce a sustained upregulation DC-SIGN, MDM were thoroughly washed, cultured for an additional 3 to 7 days in medium alone and tested for DC-SIGN. The expression levels of DC-SIGN on M2a-MDM remained consistently above control values for 7 days, the last time point studied. This prolonged expression contrasts with the more transient nature of polarization-induced changes in membrane receptor expression (Chapter 2 and 3). As previously reported, most cytokines and membrane receptors, including CD4, return to control levels 3-7 days after removal of the polarizing stimulus, either M1 or M2a. Interestingly, the number of DC-SIGN receptors per cell, as determined by MFI, continued to increase and peaked 3 days after removal of IL-4 (**Figure 4.1 C**). A similar kinetic was observed for the M2a chemokine CCL22 (Chapter 2) and suggests that M2a polarization may occur in multiple waves. Consistent with its M2a specificity, DC-SIGN+ cells in control and M2a cultures tested negative for intracellular CCL3, an M1-associated chemokine (Chapter 3) that is strongly and differentially upregulated by TNF plus IFN-γ (**Figure 4.1 D**).

4.2.2 *Anti-DC-SIGN mAb blocks HIV-1 binding to M2a-MDM.* It has been reported that compared to CD4, DC-SIGN may have a higher binding affinity for HIV-1 (29). Although the HIV-1 attached to DC-SIGN on dendritic cells can be internalized, available evidence suggests that the majority of DC-bound virus remains on the cell surface and is efficiently transmitted to T cells without internalization. To investigate the effects of DC-SIGN expression on the binding of HIV-1 to M2a-MDM, we incubated MDM with HIV-1_{BAL} (m.o.i 1) at 4°C (a condition which prevents virus entry) and measured the amount of HIV-1 p24 antigen that was attached to the cell surface after 2 hours. As expected, all three MDM populations showed an ability to bind R5 HIV-1_{BaL} (**Figure 4.2 A**) with M2a and control MDM exhibiting a greater binding affinity than M1-MDM (34.9 and 35.7 vs. 29.6 pg p24/ml respectively). Blockage of DC-SIGN with an anti-DC-SIGN mAb led to a 52.5 % (range 29% to 74%) reduction in the amount of HIV-1 bound to the surface of M2a-MDM compared to M2a that were not pre-treated with anti-DC-SIGN mAb (**Figure 4.2**). In contrast, pre-incubation with anti-DC-SIGN antibody had no significant effect on virus attachment to either control or M1-MDM, cells that express relatively low or negligible amounts of cell surface DC-SIGN (**Figure 4.2 B**). Blockage of CD4 with anti-CD4 mAb (Leu3a) led to a mean 47% (range 36% to 67%) decrease in virus attachment to control MDM but not to M1- or M2a-MDM. The failure of anti-CD4 mAb to cause significant inhibition of HIV-1 binding to M1- and M2a-MDM is consistent with the severe polarization-induced down-regulation of CD4 on these cells (**Figure 4.2 B**).

4.2.3 *Co-expression of DC-SIGN on Control and M2a MDM modulates HIV-1 entry.* Previous studies have shown that co-expression of DC-SIGN and CD4 on the surface of monocyte-derived DC and transfected cell lines (ie. CD4+/DC-SIGN+ Raji cells) can increase the efficiency of CD4-mediated entry, especially when CD4 levels are limiting (25, 26). To investigate whether the IL-4 induced expression of DC-SIGN on M2a-MDM could compensate for the polarization-induced downregulation of CD4, we infected control, M1- and M2a-MDM with HIV-1_{BaL} and measured the amount of

intracellular HIV-1 DNA that accumulated during the first 48 hours of infection. This is the time required for HIV-1 to complete a single cycle of reverse transcription, nuclear import and viral integration in macrophages (Chapter 3). Previous studies have shown the initial increase in HIV-1 DNA is an accurate marker of virus entry and reverse transcription. Viral DNA levels in M2a-MDM, as measured by real-time PCR, were comparable to those observed in unpolarized control cells indicating that there was no impairment of HIV-1 entry or early replication in M2a-MDM despite the significant downregulation of CD4 (**Figure 4.3 A**). Pre-treatment with anti-DC-SIGN mAb resulted in a 24% decrease in HIV-1 DNA levels in M2a-MDM (**Figure 4.3 B**). This finding is consistent with studies conducted on DC-SIGN$^+$ monocyte-derived DC showing that when CD4 levels are limiting, DC-SIGN facilitates the CD4-mediated entry of HIV-1 into DC (25, 26). As expected based on their low-level expression of DC-SIGN, blocking of DC-SIGN with mAb had little, or no, effect on HIV-1 DNA levels in M1-MDM. Interestingly, the blockage of DC-SIGN on control MDM led to an average 41% increase, rather than a decrease, in HIV-1 DNA (**Figure 4.3 B**). Thus, in unpolarized control MDM, expressing high levels of CD4, low levels of DC-SIGN appears to compete with CD4 for HIV-1 binding decreasing the amount of virus that is internalized.

4.2.4 *DC-SIGN inhibits virus production in a spreading M2a-MDM culture system.* Because of the sustained expression of DC-SIGN on M2a-MDM (for up to 7 days) and the importance of this receptor in mediating immune responses, we investigated whether the modulation of DC-SIGN expression contributed to the inhibition of viral replication that is characteristic of M2a activation and polarization (Chapter 3). In these experiments, control, M1- and M2a-MDM were incubated with anti-DC-SIGN or anti-CD4 blocking antibodies prior to infection with HIV-1_{BaL} (m.o.i. 0.1). Virus production was assessed at regular intervals over a 12-day incubation period by measuring the amount of HIV-1 reverse transcriptase released into the culture supernatant. HIV-1 RT levels were significantly reduced in both M1- and M2a-MDM relative to control MDM as discussed in Chapter 3

(Figure 4.4). In all three MDM populations, blocking of CD4 with anti-CD4 mAb completely inhibited RT activity over the entire 12-day infection period, underscoring the essential nature of CD4 for both virus entry and the establishment of productive infection. Interestingly, blocking DC-SIGN prior to infection led to an increase in RT activity in the supernatants of M2a-MDM **(Figure 4.4, lower panel).** Thus, although DC-SIGN enhances the CD4- mediated entry and reverse transcription of HIV-1 in the first round of infection, it appears to have a paradoxical inhibitory effect on viral replication as measured in a spreading culture system. In contrast, exposure to anti-DC-SIGN antibody had no significant effect on virus production in M1 and control cells that express only low or negligible levels of DC-SIGN **(Figure 4.4, top and middle panels).**

4.2.5 *High-level expression of DC-SIGN on M2a-MDM mediates efficient transmission of HIV-1 to T-cells.* DC-SIGN expressed on the surface of immature DC, activated B-lymphocytes and transfected B-cell lines (DC-SIGN+/CD4+-Raji cells) has been shown to strongly bind and transmit HIV-1 to T-cells. In this study, we tested whether DC-SIGN$^+$ M2a-MDM also possessed this capacity. For these studies, control and polarized M1- and M2a-MDM were incubated with X4 (T-tropic) HIV-1 (HIV-1_{IIIB}) for 2 hours, thoroughly washed to remove unbound virus and then incubated with IL-2 activated monocyte-depleted PBMC. As expected (30, 31), the X4-tropic strain used in these experiments did not replicate in control, M1 or M2a MDM. In one set of co-cultures, PBMC were separated from the adherent HIV-1-pulsed MDM (X4-MDM) after 6 h of co-culture and incubated for an additional 12 days in complete medium containing IL-2. HIV-1 transmission to "separated" PBMC target cells was quantified by measuring viral RT activity released into the culture supernatant. M2a-MDM exhibited an enhanced ability to transmit X4 virus to susceptible separated PBMC relative to control and M1-MDM **(Figure 4.5A).** Blockage of DC-SIGN receptors on MDM before pulsing with X4 virus resulted in an 84.1% to 93.7% reduction in HIV-1 transfer from M2a-MDM to T cells indicating that all, or nearly all, of the transmission was mediated by DC-SIGN **(Figure**

4.5 B, left column). In another set of experiments, X4-MDM were maintained in co-culture throughout the entire 12-day incubation period. Unexpectedly, in these long-term co-cultures only low levels of HIV-1 transmission were observed for M2a-, as well as M1- and control MDM (**Figure 4.5A**). Blocking DC-SIGN resulted in decreased transmission from M2a, but not from M1 or control MDM, indicating that the small amount of viral transfer that did occur during prolonged exposure to X4-M2a-MDM was also mediated by DC-SIGN (**Figure 4.5 B, right column**). Collectively, these data provide evidence for a strong association between M2a-induced expression of DC-SIGN and enhanced *trans*-transmission of HIV-1, especially in short-term (6 h) co-cultures.

4.3 DISCUSSION

We have previously reported that cytokine-induced polarization of MDM into M1 (pro-inflammatory) and M2a (anti-inflammatory) macrophages leads to an inhibition of productive HIV-1 infection, but that the degree of viral restriction and the phenotypic changes associated with these responses are clearly different (Chapter 3). In this chapter we describe an additional mechanism related to DC-SIGN expression that may be of direct relevance to the HIV-1 pathogenesis in M2a environments. As discussed in Chapter 2, DC-SIGN, a molecule that binds and transmits HIV-1, was the only surface membrane receptor that showed a clear-cut divergent (or inverse) pattern of expression on M1- (down-regulation), M2a- (upregulation) and control MDM. As described in this Chapter, the expression of DC-SIGN on M2a-MDM changed both the infection and transmission properties of these cells.

The strong association between M2a polarization and the upregulation of DC-SIGN suggests that this C-type lectin may play an important role in the M2/Th2 axis of immunity. This concept is strengthened by studies showing that IL-13, another type 2 cytokine, also upregulates DC-SIGN on MDM and that the treatment of breast milk macrophages with IL-4 leads to the induction of DC-SIGN (32, 33). *In vivo*, DC-SIGN has been detected on specialized macrophages of the lung and the placenta. Both of these tissues are T_H2 environments with high levels of anti-inflammatory cytokines (32, 34-36). Elevated levels of DC-SIGN have also been detected on DC in allergic nasal polyps, a well-characterized T_H2-mediated disorder (32). In the gastrointestinal tract, the expression of DC-SIGN on DC has been linked to an increase in the IL-10:IL-12 ratio and a switch to a more T_H2-type environment (37).

In this Chapter, I show that the level of DC-SIGN expression may play a key role, not only in virus binding and entry, but also in the replication and transmission of HIV-1 to T cells and that these functions are closely associated with CD4 expression. I found that in M2a-MDM expressing low levels of CD4, DC-SIGN co-expression significantly enhanced

the binding, internalization and reverse transcription of HIV-1. These results confirm and extend previous studies in DC and transfected Raji cells showing that, when CD4 levels are low, DC-SIGN co-expression can increase the efficiency of CD4 mediated viral entry. Collectively, these data indicate that, in the presence of high levels of DC-SIGN, only low levels of CD4 expression are required for the efficient viral entry and reverse transcription. Blocking DC-SIGN on the surface of M2a-MDM led to significant decreases in both HIV-1 binding and viral DNA accumulation 48 hours post-infection. The observation that 47.5% of the binding to M2a-MDM was CD4/DC-SIGN-independent suggests that, as previously proposed (38, 39), additional C-type lectin receptors, such as the mannose receptor (MR) may contribute to HIV-1 binding to MDM.

In contrast, a different scenario was observed in control MDM expressing moderate levels of CD4 and low levels of DC-SIGN. As would be expected, the binding of HIV-1 to the surface of these cells was significantly reduced (47%) by anti-CD4, but not by anti-DC-SIGN, blocking antibodies. As proposed for M2a-MDM, the remaining 53% of the binding to control MDM may be due to HIV-1 capture by MR and other C-type lectins. An unexpected finding in control cultures was that the blocking of DC-SIGN on these cells led to a 41% increase, rather than a decrease, in virus entry and HIV-1 DNA synthesis. Although the mechanisms are not known, this finding suggests that in control MDM, low levels of DC-SIGN may be competing with CD4 for the binding and capture of HIV-1 virions. The blocking of HIV-1 infection by anti-CD4 antibodies, in all three MDM populations (control, M1 and M2a) indicates, that even under conditions of high DC-SIGN expression, the CD4 receptor is still essential for virus entry and HIV-1 DNA synthesis.

As reported in Chapter 3, and re-confirmed in this Chapter, virus production was strongly inhibited by both M1 and M2a polarization. In M1-MDM, the suppression of productive infection was expected, given the dramatic polarization-induced down-regulation of both CD4 and DC-SIGN on the surface of these cells. However, in M2a-MDM, suppression of virus production occurred despite a DC-SIGN-mediated

enhancement of viral binding, internalization and HIV-1 DNA synthesis. As previously discussed, these findings suggest that in M1-MDM, the block in HIV-1 infection occurs at the level of viral entry while in M2a-MDM virus production is restricted at a late step in the viral life cycle. Of particular interest in this study, was the finding that pre-treatment of M2a-MDM with anti-DC-SIGN could partially overcome the late-stage block in M2a infection. The mechanisms underlying this reversal of inhibition are not known but may be due to a DC-SIGN-mediated signaling event (potentially Raf-1 upregulation of IL-10) that was either initiated or inhibited by the interaction with anti-DC-SIGN mAb.

The strong, clear-cut association between the expression of DC-SIGN and the ability of M2a-MDM to bind and rapidly transfer HIV-1 to PBMC (ie. within 6 h) suggests that, as observed for dendritic cells, DC-SIGN may play an important but restricted role in the *trans*-transmission of HIV-1 from M2a macrophages to CD4+ T cells. Consistent with this observation, I found that pre-incubation of M2a-MDM with anti-DC-SIGN antibodies led to a near-complete (84%-94%) block in HIV-1 transmission. The reason why short-term exposure to X4-pulsed MDM led to efficient viral transmission, while long-term (12 day) exposure abolished transmission are not known but may be related to the complexity of the MDM-PBMC co-culture system, including a potential upregulation of inhibitory cytokines and chemokines by polarized MDM. An understanding of the factors limiting virus production in these co-cultures may lead to insights for the control of cell-to-cell transmission.

In summary, the restricted expression of DC-SIGN on M2a-MDM correlates well with *in vivo* studies showing that DC-SIGN is expressed on a limited subset of tissue macrophages found in T_H2 environments. The dichotomous effect of DC-SIGN on HIV-1 entry (enhancement) and virion production (inhibition) in M2a-MDM suggests that DC-SIGN may play a role in the establishment of viral reservoirs in cells of the M2a phenotype. Of particular interest in this regard, is the finding that different levels of DC-SIGN and CD4+ co-expression may a pivotal role in the regulation of *cis*- versus *trans*-

infection. Finally, DC-SIGN+ macrophages may facilitate *in vivo* transmission of HIV-1 at mucosal membranes, especially in tissues with a high concentration of macrophages and a predominantly M2/T_H2 environment.

4.4 FIGURE LEGENDS

Figure 4.1 Impact of M1 and M2a polarization on the expression of DC-SIGN. **A.** Changes in DC-SIGN expression on the plasma membrane of M2a-MDM after 18 hour polarization. Cells were labeled with anti-DC-SIGN mAb and analyzed by flow cytometry. Results from a single donor are representative of 6 donors tested. **B.** Upregulation of DC-SIGN on M2a-MDM as determined by microscopic evaluation. Results shown are for a single representative donor of 2 donors analyzed. 800-1200 cells were counted per donor in triplicate for each MDM condition. **C.** The kinetics of DC-SIGN expression at an individual cell level were assessed on control and polarized MDM after 18 hours, and at day 3 and 7 post-stimulation. Results are reported as the mean values from 4 individual donors. **D.** Evaluation of DC-SIGN as a marker of M2a polarization. Control, M1- and M2 MDM were labeled with anti-DC-SIGN, fixed, permeabilized and labeled intracellularly with CCL3 Ab. Results are reported as the average percentage of positive cells (DC-SIGN+ CCL3-, DC-SIGN-CCL3+ or DC-SIGN+CCL3+) from 4 individual donors.

Figure 4.2 Binding of HIV-1_{BaL} to M1- and M2a-MDM correlates with the differential expression of CD4 and DC-SIGN. **A.** HIV-1 binding to control ($CD4^{high+}DC\text{-}SIGN^{low+}$), M1- ($CD4^{low+}DC\text{-}SIGN^{low+}$) and M2a- ($CD4^{low+}$ $DC\text{-}SIGN^{high+}$) MDM. Virus attachment was determined by measuring the amount of p24 antigen bound to the cell surface. **B.** Anti-DC-SIGN mAb inhibits virus binding to M2a-MDM, but not to control or M1-MDM, whereas pre-incubation with anti-CD4 mAb, significantly inhibits HIV-1 to control MDM expressing high levels of CD4. Values were normalized relative to non-antibody treated MDM and results are reported as the mean fold-change in p24 antigen in 3 independent replicates performed on MDM derived from 8 different donors.

Figure 4.3 Dichotomous effects of DC-SIGN on HIV-1 entry and replication in M2a- vs control and M1-MDM. **A.** Blockade of DC-SIGN with anti-DC-SIGN mAb decreased the accumulation of HIV-1 DNA in M2a-MDM, but not in M1-MDM. Conversely, blocking of DC-SIGN enhanced HIV-1 DNA levels in control MDM. MDM

were pre-incubated with anti-DC-SIGN mAb for 2 h at 4°C. Cultures were infected with HIV-1_{BaL} and harvested after 48 h. At the end of this time, the cells were lysed and the amount of viral DNA in each lysate was quantified using a real-time PCR assay that measures total HIV-1 *gag* DNA. Results shown are representative of 1/6 donors, all of whom had similar DNA profiles. **B.** Data shown as the fold change (increase or decrease) in HIV-1 DNA accumulation in the presence and absence (nil) of pre-treatment with anti-DC-SIGN antibody. Results shown represent the average of 2 separate experiments performed on a total of 6 independent MDM donors.

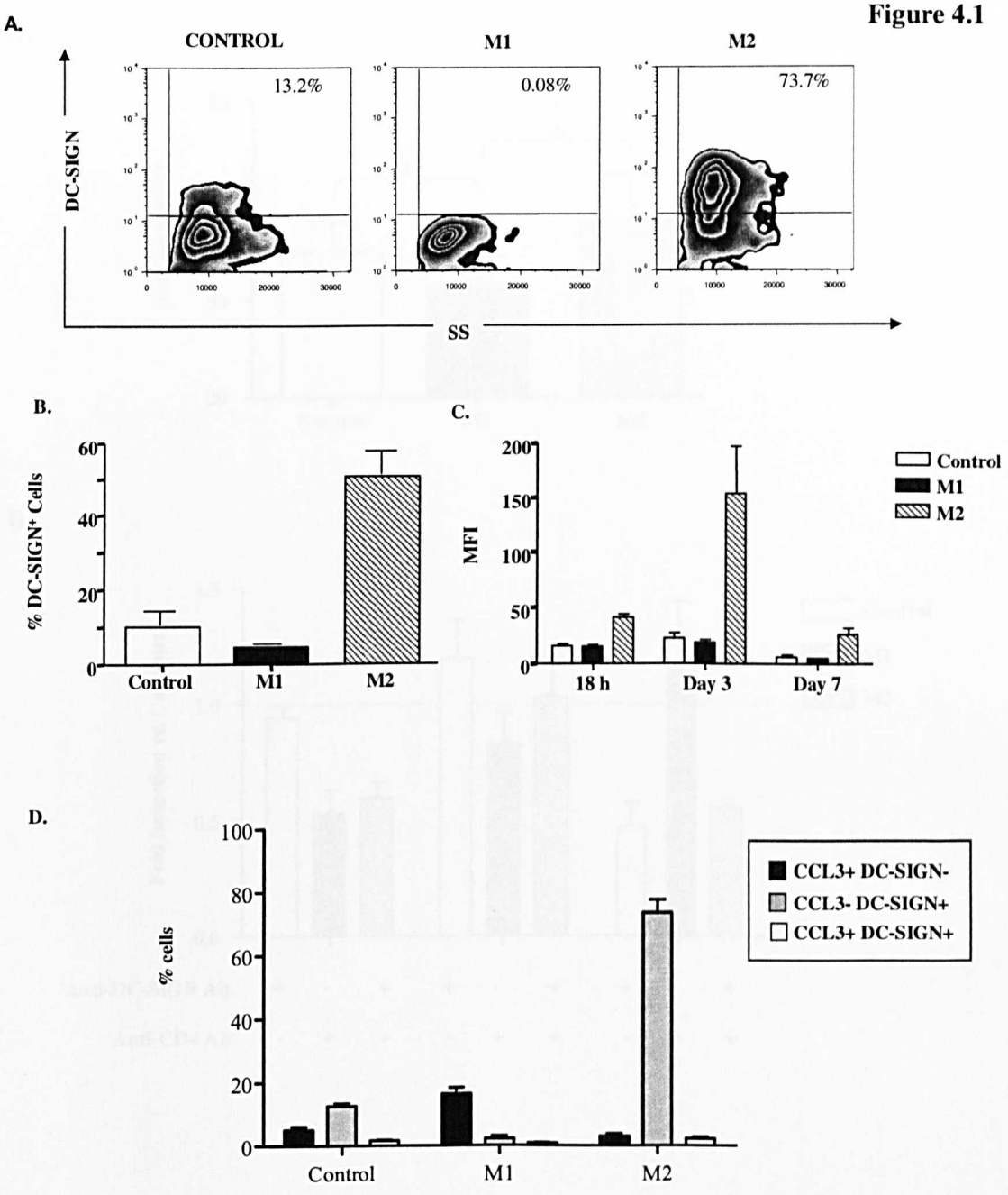
Figure 4.4 Blocking of DC-SIGN enhances virus production in M2a- but not in M1- or control MDM. Untreated MDM and MDM pre-treated with either anti-CD4 or anti-DC-SIGN mAb were infected with HIV-1_{BaL} at an m.o.i. of 0.1. Virus production in control (upper panel), M1- (middle panel) and M2a- (lower panel) MDM was monitored over a 12-day culture period by measuring the amount of RT activity released into the culture medium. As shown, anti-CD4 antibodies completely inhibited productive infection in all 3 MDM populations. Results represent the mean of 3 independent replicate assays ± standard deviations. Similar results were obtained for MDM derived from 4 different donors.

Figure 4.5 DC-SIGN-mediated transmission of HIV-1 from M2a-MDM to permissive PBMC is rapid and efficient. A. Effects of short- vs. long-term exposure to MDM pulsed with X4-tropic virus. Control and differentially polarized M1- and M2a-MDM were incubated with HIV-1_{IIIB} for 2 h at 37°C and then washed to remove unbound virus. The HIV-1-pulsed MDM were then co-cultured with IL-2-activated PBMC for 6 h, at which time the MDM were removed from PBMC culture (short-time exposure). Alternatively, the HIV-1-pulsed MDM were retained in the PBMC co-culture throughout the 12-day period (long-term exposure), a condition which suppressed virus production and/or spreading, and obscured the transmission differences between the 3 MDM populations. HIV-1 transmission was determined by measuring RT activities in the PBMC

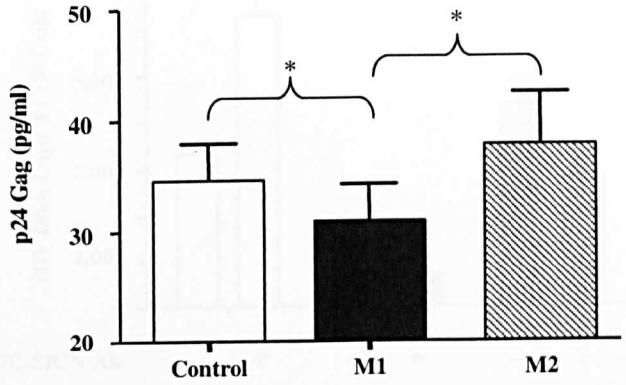
supernatants. Results are expressed as the percent transmission relative to control MDM (100%) ± standard deviations of 3 independent wells from 4 individual donors. **B.** Pre-incubation with anti-DC-SIGN mAb interferes with the ability of M2a-MDM to bind and transmit HIV-1 to PBMC. Study design was the same as discussed above, except that DC-SIGN receptors on MDM were blocked with mAB before exposure to X4 HIV-1. Results are expressed as the average from 4 individual donors.

4.5 FIGURES

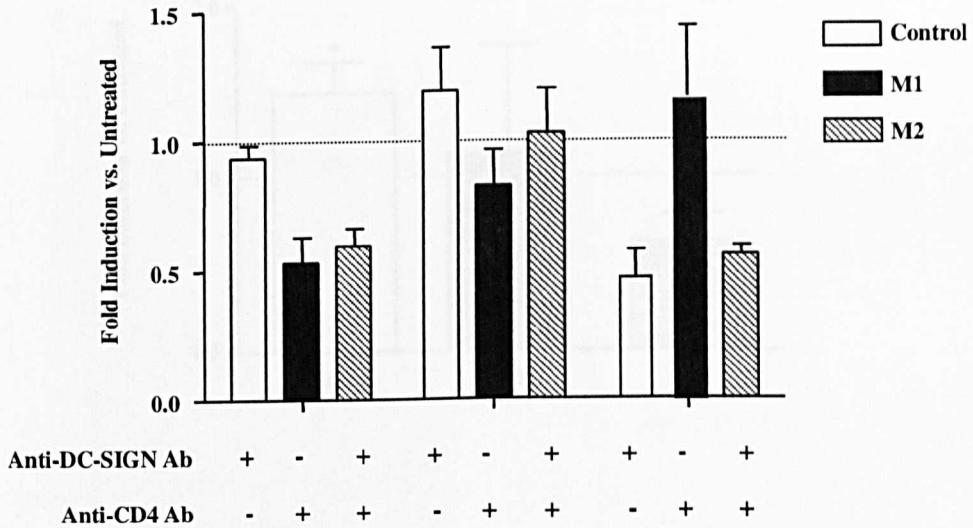
Figure 4.1



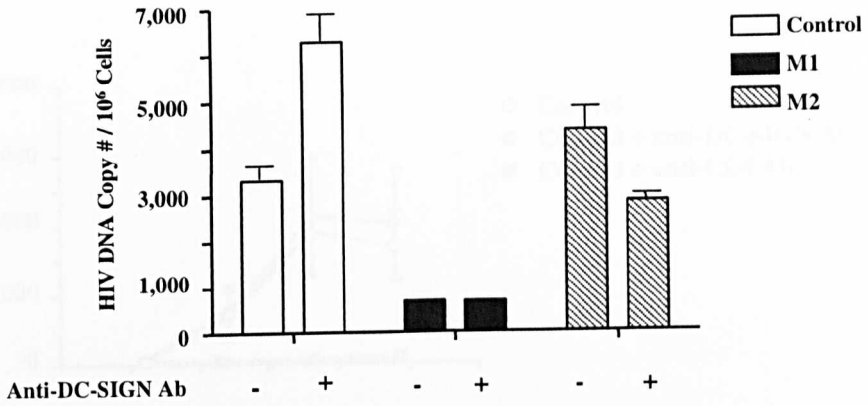
A.


50
40
30
20
p24 Gag (pg/ml)
*
*
Control
M1
M2

B.


1.5
1.0
0.5
0.0
Fold Induction vs. Untreated
Control
M1
M2
Anti-DC-SIGN Ab + - + + - + + - +
Anti-CD4 Ab - + + - + + - + +

A.


7,000
5,000
3,000
1,000
0
HIV DNA Copy # / 10⁶ Cells
Control
M1
M2
Anti-DC-SIGN Ab
-
+
-
+
-
+

B.

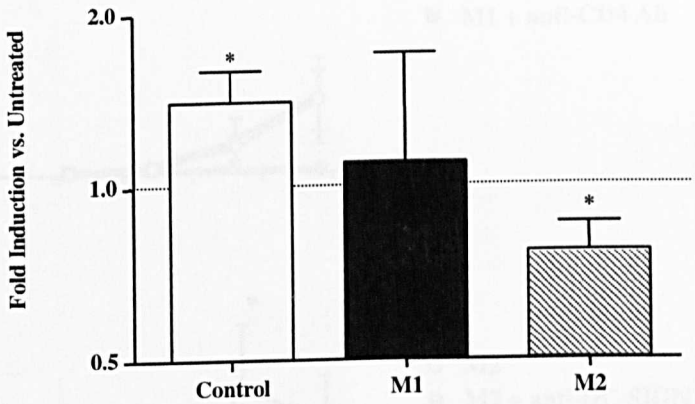

2.0
1.0
0.5
Fold Induction vs. Untreated
*
*
Control
M1
M2

Figure 4.4

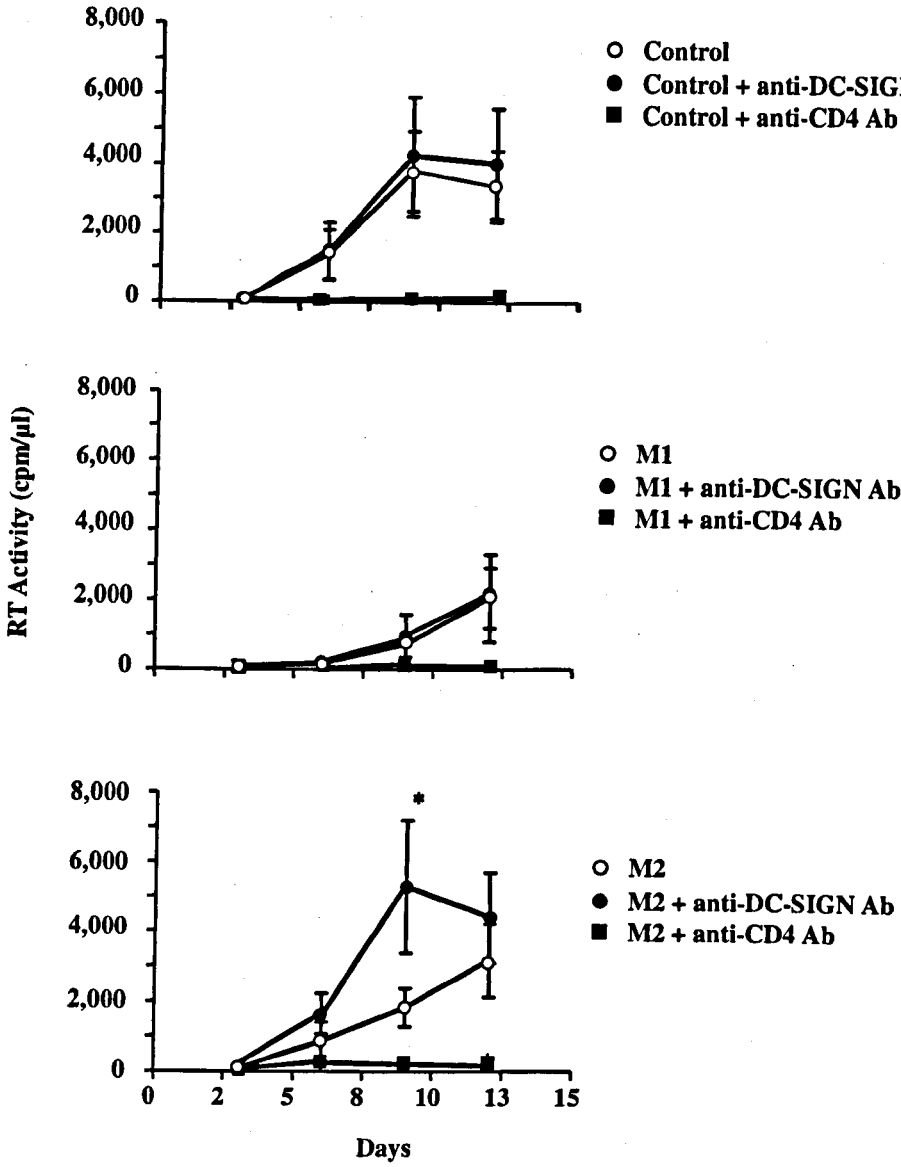
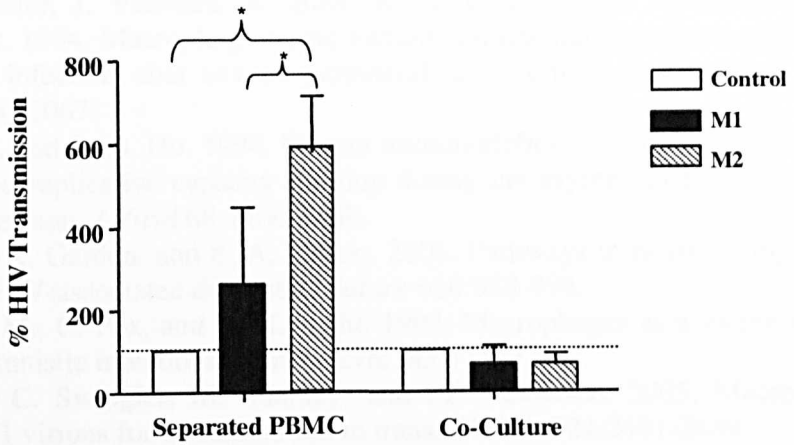
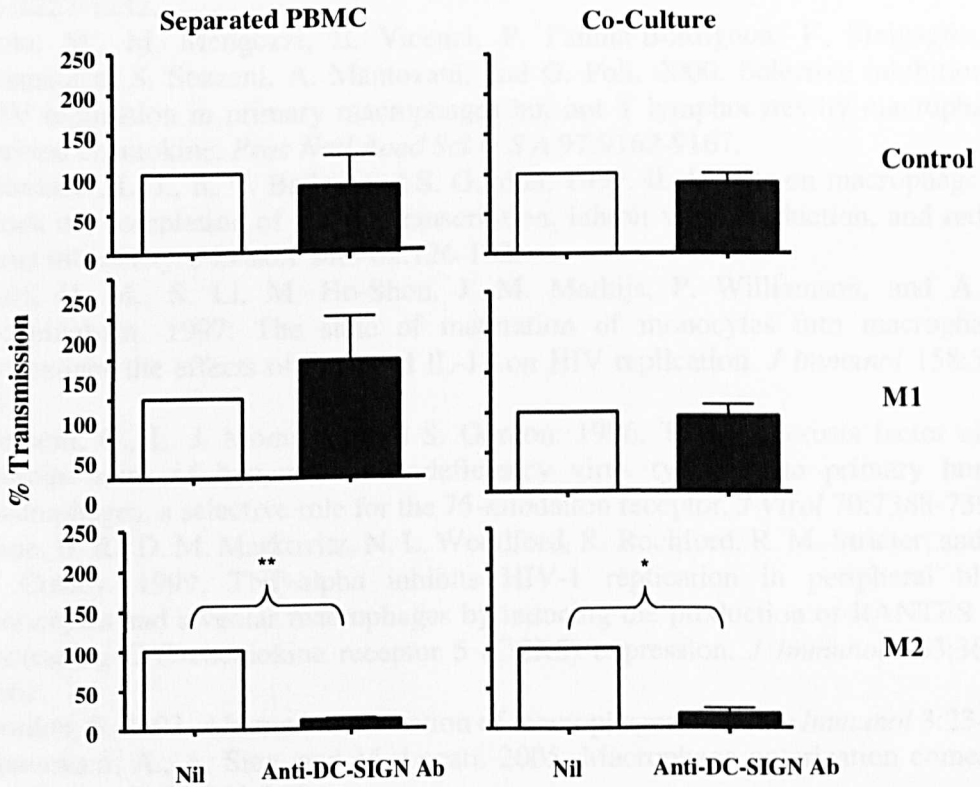

8,000
6,000
4,000
2,000
0
Control
Control + anti-DC-SIGN Ab
Control + anti-CD4 Ab
RT Activity (cpm/µl)
M1
M1 + anti-DC-SIGN Ab
M1 + anti-CD4 Ab
M2
M2 + anti-DC-SIGN Ab
M2 + anti-CD4 Ab
*
0
2
5
8
10
13
15
Days

Figure 4.5

A.


% HIV Transmission
800
600
400
200
0
*
*
Control
M1
M2
Separated PBMC
Co-Culture

B.


Separated PBMC
Co-Culture
% Transmission
250
200
150
100
50
0
Control
M1
M2
**
*
Nil
Anti-DC-SIGN Ab
Nil
Anti-DC-SIGN Ab

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CHAPTER 5
MONOCYTE ACTIVATION IN TREATMENT NAÏVE SOUTH AFRICAN AIDS PATIENTS IS INDEPENDENTLY DRIVEN BY HIV-1 AND MICROBIAL TRANSLOCATION

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5.0 INTRODUCTION

HIV-1 transmission and pathogenesis are intimately related to the activation state of the host immune system (1-3). Although immune activation is essential to mount an effective response, it also provides an environment that drives HIV-1 replication and disease progression (4). Increased levels of immune activation correlate with $CD4^+$ T cell depletion (5-8) and chronic $CD8^+$ T cell activation (9). In patients with advanced HIV-1 infection, activation is a stronger predictor of disease progression than plasma viremia (1). In addition to enhancing HIV-1 replication (9-12), chronic immune activation leads to exhaustion and dysfunction of the host immune system (13-17). Thus, understanding the factors that drive chronic immune activation may provide new insights into the development of more effective therapeutic strategies.

Recently, it has been suggested that the massive $CD4^+$ T cell depletion that occurs in the gastrointestinal tract (GIT) during acute HIV-1 infection leads to mucosal immune dysfunction in association with increased microbial translocation, systemic immune activation and progression to AIDS (18-20). In support of this hypothesis, sooty mangabeys naturally-infected with simian immunodeficiency virus (SIV) do not show any evidence of sustained microbial translocation and do not develop an AIDS-like illness despite having high viral loads and mucosal $CD4^+$ T cell depletion (21). In contrast, pathogenic SIV infection in rhesus macaques leads to sustained microbial translocation, high-level chronic immune activation and progressive disease, mimicking the typical course of HIV-1 infection in humans (22). Macaque infection is also associated with alterations in innate immunity including the activation and proliferation of natural killer (NK) cells, high levels of dendritic cell (DC) activation and an exacerbated IFN-α response (23).

In addition to $CD4^+$ T lymphocytes, cells of the mononuclear phagocyte lineage also play an important role in the activation-induced pathogenesis of HIV-1 infection (24). Mononuclear phagocytes are the primary targets of LPS-induced activation and are key regulators of inflammatory and anti-inflammatory responses. They respond to a wide range of environmental stimuli and depending on the stimulus can be polarized into functionally distinct "immune regulatory/inflammatory" (M1) or "tissue remodeling" (M2) phenotypes (25). In addition, a subset of inflammatory monocytes expressing high levels of CD16 (the Fc receptor γ III, FcRγIII) is markedly upregulated in HIV-1 infected patients, especially in patients with advanced disease (26, 27). *In vitro,* $CD14^+CD16^+$ monocytes are more susceptible to HIV-1 infection than CD16-negative cells and they are an important source of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) (28). $CD14^+CD16^+$ monocyte-derived-macrophages (MDM) are more efficient at activating resting T cells and *in vitro* conjugates formed between $CD14^+$CD16+ MDM and T cells are major sites of virus production (29, 30). *In vivo*, $CD14^+CD16^+$ monocytes have been postulated to play a key role in HIV-1 infection of the central nervous system (CNS) by transporting virus into the brain and, upon differentiation into perivascular macrophages, by releasing neurotoxins that damage the underlying tissue (31-34). Although the activation of mononuclear phagocytes appears to play an important role in HIV-1 pathogenesis, the mechanisms driving the activation of these cells are not fully understood.

In the present study, 1 investigated the relationships between markers of monocyte activation, HIV-1 viremia and LPS (an indicator of microbial translocation) in a cohort of $HIV\text{-}1^+$ ART-naïve African patients with no evidence of active opportunistic or enteric co-infections. Higher levels of monocyte activation were detected in $HIV\text{-}1^+$ relative to uninfected South Africans, and in South Africans compared to North Americans and Europeans. Two distinct activation phenotypes were identified: one statistically linked to microbial translocation, the other to HIV-1 replication. Plasma levels of LPS were positively correlated with circulating sCD14 and TNF-α, suggesting that up-regulation of

these markers was causatively linked to microbial translocation. In contrast, the upregulation of CCL2 and increased frequency of $CD14^+CD16^+$ monocytes were positively correlated with HIV-1 viremia, suggesting that HIV-1 rather bacterial products are the primary determinant driving the expansion and potential recruitment of $CD14^+CD16^+$ monocytes to sites of tissue inflammation. Elevated levels of CCL2 and $CD14^+CD16^+$ monocytes have been previously linked to the development of HIV-1-associated dementia (HAD) (35, 36). These findings may have important implications for preventing the deleterious effects of immune activation and enhancing the efficacy of antiretroviral therapy.

5.1 MATERIALS AND METHODS

5.1.1 *Study cohorts.* HIV-1-infected South African patients with peripheral blood CD4 counts of <250 cells/μl were recruited at the Comprehensive Care, Management and Treatment (CCMT) Clinic of the Tshwane District Hospital in Pretoria, South Africa. Baseline $CD4^+$ T cell counts, plasma HIV-1 RNA, full blood counts, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) testing was conducted as part of their routine clinical assessment. Patients were considered for the study if they were ≥18 years of age, were ART-naïve, tested negative for TB in sputum smears stained for acid fast bacilli (AFB), had no evidence of an active bacterial or opportunistic infections (OIs) and had not taken antibiotics for 6 months prior to sampling. Bacterial, cytomegalovirus (CMV) and parasitic infections (including *Cryptosporidium*) of the small intestine were excluded based on two negative stool cultures (MCS) and histological examination of biopsy samples. A total of 19 African patients (11 females, 8 males) with stage III or IV disease (WHO classification) (37) were recruited into the study. As summarized in Table 1, these patients had a mean age of 35 years (SD ± 9.3, range 24-58), a mean CD4+ T cell count of 108 cells/μl (SD ± 97, range 6-378) and a mean plasma viral load of 4.5 $\log_{10}$ HIV-1 RNA copies/ml (SD ± 1.1; range 1.7-6.1). Since previous studies have suggested that OIs may be largely responsible for the expansion of $CD14^+CD16^+$ monocytes (38, 39), we also recruited and performed monocyte subset analyses on a subset of patients with active OIs (n = 26). This latter group of patients was also ART-naïve and presented with a wide variety of bacterial, viral and fungal co-infections. Ten healthy HIV-1-negative volunteers were recruited from an outpatient clinic. The protocol was approved by the Ethics Committee of the University of Pretoria, South Africa and each participant gave written informed consent.

5.1.2 *Plasma viremia.* Plasma was isolated from EDTA blood tubes by spinning at 1600 rpm for 10 minutes. After separation, plasma was stored in 0.5 ml aliquots at -80°C

until needed for RNA isolation. RNA was extracted using a guanidinium-silica method (Magnetic Extraction Reagent kit from BioMerieux Inc) with no modifications to the protocol. HIV-1 RNA levels in plasma were quantified using the Nuclisens Easy Q HIV-1 v.1.2 kit, also with no modifications to the protocol. As 1.0 ml of plasma was used for all viral load determinations, the lower limit of detection was 40-50 copies/ml; the upper limit was >500 000 copies/ml.

5.1.3 *Determination of $CD4^+$ T cell counts and monocyte subsets.* $CD4^+$ T-cell counts and percentages were evaluated using a cocktail of anti-human murine CD45 (clone J.33) and CD4 (clone 13B8.2) monoclonal antibodies (mAb) (Beckman Coulter, Fullerton, California, USA). $CD14^+CD16^+$ monocyte percentages were determined using murine mAbs directed against human CD14 (clone RMO52) and CD16 (clone 3G8) molecules (Beckman Coulter). These mAb, used as specified by the manufacturer, were added to 100 µl of whole blood collected in an EDTA tube. The samples were vortexed, incubated in the dark for 10 minutes at room temperature and then placed in TQ-Prep (Beckman Coulter) in order to lyse the red blood cells and fix the leukocytes. Absolute counts and percentages were determined using a single platform protocol and 100 µl of Flow Count Beads (Beckman Coulter) on an FC500 flow cytometer (Beckman Coulter). Monocytes were analyzed based on forward and side scatter properties and on the expression of $CD14^+$ and $CD16^+$ markers.

5.1.4 *Quantification of soluble activation markers.* Plasma LPS levels were quantified by the Limulus Amebocyte Lysate (LAL) assay QCL-1000 (Lonza, Valais Switzerland). Samples were diluted 1:3 with LAL Reagent Water, inactivated for 30 minutes at 65°C and tested in a 96 well microplate. Plasma levels of soluble CD14 (sCD14) were measured using an ELISA assay (R&D Systems, Minneapolis, MS). Cytokine and chemokine levels in plasma [interferon-γ (IFN-γ), TNF-α, IL-4, IL-6, IL-12, IL-10, CCL2, CCL3, CCL4 and CCL5 CXCL10] were quantified using customized Bio-Plex plates (Bio-Rad, Hercules, CA). For cytokine/chemokine analyses, plasma samples

were diluted 1:5 with Human Serum Diluent (Bio-Rad) prior to quantification. All assays were performed according to manufacturer's instructions.

5.1.5 *Statistical analysis.* Prism 5 from GraphPad Software (La Jolla, CA) was used for statistical analyses. Results are reported as mean values $\pm$ SD or median values with ranges. Analysis of variance was determined using the Mann-Whitney U test. Spearman correlations (r and p values) were calculated to determine the relationships between HIV viremia, plasma levels of LPS and activation markers.

5.2 RESULTS

5.2.1 *African AIDS patients without OI show increased plasma levels of type 1/type 2 cytokines and normal (CCL3, CCL4) or reduced (CCL5) expression of CCR5-binding chemokines.* In order to investigate the extent and pattern of immune activation in our cohort of African patients with advanced HIV-1 disease and no OIs, we measured the plasma concentrations of CXCL10, CCR5-binding chemokines (CCL3, CCL4, CCL5), type 1 (IFN-γ, IL-12) and type 2 (IL-4, IL-10) cytokines. These values were then compared to those measured in uninfected Africans. Plasma levels of all type 1 and type 2 cytokines were consistently higher (2.5 to 4.6-fold) in AIDS patients compared to the uninfected controls (p=0.006 to 0.012) suggesting that HIV-1 causes a generalized up-regulation of cytokines (**Table 2**). Although less dramatic, CXCL10, a chemokine that plays an important role in the enhancement of HIV-1 encephalitis (40, 41) was also up-regulated (1.8-fold; p=0.02) in HIV-1+ relative to the uninfected controls. No significant changes were detected in the expression of the CCR5-binding chemokines, CCL3 and CCL4, between HIV-1$^+$ and uninfected individuals (**Table 2**). In contrast, plasma levels of CCL5 were downregulated in HIV-1$^+$ individuals relative to uninfected controls (p=0.0096) (**Table 2**). No significant associations were detected between plasma levels of chemokines/cytokines and either CD4$^+$ T cell counts or HIV-1 viremia.

5.2.2 *Markers of immunological activation are higher in Africans vs. non-Africans.* When compared to published data from cohorts in North America and Europe, African AIDS patients had elevated (2 to 5-fold increases) levels of both type 1 (IFN-γ, IL-12) and type 2 (IL-4, IL-10) cytokines, in addition to increased expression of CCL3, CCL4, CCL5 and CXCL10 (**Table 2**). Several analytes (IL-4, IL10, CCL4, CCL5 and CXCL10) were also expressed at higher levels in healthy HIV-1$^-$ Africans relative to uninfected American and European controls, suggesting that even in the absence of HIV-1, Africans have an increased "background" level of activation (**Table 2**) (42, 43).

5.2.3 *AIDS in Africans is characterized by a generalized upregulation of cytokines and an increase in the IL-10:IL-12 ratio.* To establish whether HIV-1 infection in Africa was associated with a polarization of cytokine profiles, we measured differences in the IL-4/IFN-γ (related primarily to T cells) and IL-10/IL-12 (produced primarily by mononuclear phagocytes) ratios of HIV-1$^+$ vs. healthy controls. Interestingly, we observed that HIV-1 infection did not alter the T-cell associated ratio (IFN-γ:IL-4) (**Figure 5.1**). However, there was a 0.8-fold increase (p=0.034) in the IL-10:IL-12 ratio in HIV-1$^+$ compared uninfected controls. Thus, advanced HIV-1 disease was associated with a significant increase in the expression of IL-10, an anti-inflammatory and immunosuppressive cytokine produced primarily by mononuclear phagocytes (**Figure 5.1**) (44).

5.2.4 *Increased frequency of circulating CD14$^+$CD16$^+$ monocytes in the absence of active OIs and enteric parasites.* Previous studies have shown that the expansion of CD14$^+$CD16$^+$ monocytes is most pronounced during late-stage disease, especially in patients with OIs and AIDS-associated dementia (27). In this study, a statistically significant increase in the frequency of circulating CD14$^+$CD16$^+$ monocytes was detected in ART-naïve AIDS patients without OIs compared to controls (mean 26% versus 13%; p=0.002) (**Table 3** and **Figure 5.2**). As observed for cytokines and chemokines, the values for both infected and uninfected Africans were higher than those published for non-African populations (26% vs 16% for infected and 13% vs 6.5% for uninfected controls) (**Table 3**). Interestingly, no significant difference was detected in the mean frequency of CD14$^+$CD16$^+$ monocytes among African AIDS patients with or without active opportunistic disease (n=26), (26% versus 27.4%, p=0.752) (**Figure 5.2**). However, we did observe that CD14$^+$CD16$^+$ monocyte levels were more variable among patients with overt opportunistic disease compared to those without co-infections. This may be a consequence of the broad spectrum of bacterial (n=10), viral (n=9) and fungal (n=7) co-infections in this group of patients (**Figure 5.2**).

5.2.5 *AIDS is associated with high levels of monocyte activation in African patients.* We next measured the plasma levels of several key markers of monocyte activation. Both HIV-1 positive and seronegative individuals had detectable plasma levels of sCD14, TNF-α, IL-6 and CCL2. All four markers were significantly upregulated (>2-fold; p=0.0003 to 0.03) in the HIV-1$^+$ vs. uninfected controls with IL-6 and CCL2 showing the most striking mean increases (2.7- and 3.9-fold, respectively) (**Table 3** and **Figure 5.3**). TNF-α, IL-6 and CCL2 levels were higher in African compared to European and North American populations, for both infected and uninfected controls (**Table 3**). High levels of CCL2, but not of sCD14, TNF-α or IL-6, were statistically associated with an increased frequency of CD14$^+$CD16$^+$ monocytes (**Figure 5.4**). This finding is consistent with studies showing that CD14$^+$CD16$^+$ monocytes are an important source of CCL2 (29). No correlations were found between these markers and the patients' CD4$^+$ T cell count suggesting that there is no direct relationship between monocyte activation and the loss of CD4$^+$ T cells, at least not in our cohort of patients with advanced HIV-1 infection.

5.2.6 *Monocytes are differentially activated by HIV-1 and microbial translocation.* In order to establish whether HIV-1 viremia and/or bacterial products were correlated with high levels of monocyte activation, I investigated the relationships between plasma LPS (a reliable marker of microbial translocation) (19, 35). HIV-1 viremia and markers of monocyte activation. High levels of plasma viremia were moderately correlated with an increased frequency of CD14$^+$CD16$^+$ monocytes (r=0.56; p=0.01) and, to a lesser extent, with circulating levels of CCL2 (r=0.49; p=0.03). In contrast, no positive or negative correlations were detected between HIV-1 viremia and the other activation markers (ie. sCD4, TNF-α or IL-6) (**Figure 5.5**).

As expected,(19, 35) circulating levels of LPS were higher in HIV$^+$ patients vs. HIV$^-$ controls (1.059 vs. 0.465 respectively, p=0.007, **Figure 5.6A**). Unlike viremia, circulating levels of LPS were positively correlated with both sCD14 (r=0.53; p=0.02) and

TNF-α (r=0.62, p=0.005), but not with IL-6, CCL2 or circulating levels of $CD14^+CD16^+$ monocytes (**Figure 5.6B**). No correlations were detected between LPS and the expression of any of the other cytokines investigated in our study. Collectively, these correlations suggest that HIV-1 is the primary determinant fueling the expansion and recruitment of $CD14^+CD16^+$ monocytes, while microbial translocation may be main factor driving the up-regulation of TNF-α and the release of sCD14 from mononuclear phagocytes.

5.3 DISCUSSION

In this chapter, I investigated the relationships between markers of monocyte activation, plasma LPS, HIV-1 viremia and levels of cytokines/chemokines in a homogenous cohort of African AIDS patients without OIs. High levels of monocyte activation (as measured by plasma concentrations of TNF-α, IL-6 and CCL2, and an increase in the number of $CD14^+CD16^+$ monocytes) were detected in both $HIV\text{-}1^+$ individuals and uninfected African controls. Although a generalized increase in cytokines was detected in both cohorts, the increase was significantly greater in $HIV\text{-}1^+$ vs uninfected controls. There was no clear-cut bias towards a type 1 or type 2 immune response. However, we did note a disproportionate increase IL-10 relative to IL-12, especially in HIV-1+ individuals. Increased circulating levels of CXCL10 and decreased levels of CCL5 (but not of other CCR5-binding chemokines) were typically observed in $HIV\text{-}1^+$ patients. No correlations were detected between these markers and CD4+ T cell counts or viremia. Interestingly, monocyte activation appeared to involve the induction of two distinct pathways - an HIV-1-associated pathway involving the upregulation of CCL2 and $CD14^+CD16^+$ monocytes and an LPS-associated pathway linked to the upregulation of sCD14 and TNF-α.

These results extend previous studies showing that the frequency of activated (HLA-DR+) CD4+ T cells and the ratio of memory (CD45RO+) to naïve (CD45RA+) CD4+ T cells is increased in $HIV\text{-}1^+$ and healthy Africans compared to non-African populations (45). They also confirm and extend studies showing elevated levels of eosinophilia, immunoglobulin, IL-6, TNF-α, soluble TNF receptors and CCR5 expression in African populations (43, 46-49). African activation profiles have, for a long time, been attributed to a high prevalence of parasitic and non-parasitic infections (including helminth infections, malaria and tuberculosis) and it has been suggested that these infections may contribute to the explosive nature of the AIDS epidemic in Africa (43, 45, 47, 50). It has

also been proposed that, because of the high rates of helminth infections, Africans might have a more prominent type-2 immune response (43). In our study, increased "background" levels of type 1 (IFN-γ, IL-12) and type 2 (IL-4, IL-10) cytokines were detected in the absence of active tuberculosis or enteric parasites, in healthy as well as HIV-1$^+$ Africans. There was no significant difference in the T_H1:T_H2 cytokine ratio (IFN-γ:IL-4) in infected vs. uninfected Africans, a profile that is suggestive of a generalized rather than a polarized activation profile. A similar lack of polarization has been reported for HIV-1$^+$ individuals living in the UK and North America (51, 52). In our study, a higher IL-10:IL-12 ratio was detected in HIV-1$^+$ vs. healthy Africans. Since both cytokines are produced primarily by mononuclear phagocytes (53), this suggests that that there may be a shift in the innate arm of the immune system towards a more immunosuppressive environment that is driven by IL-10 production. Whether the heightened state of immune activation in Africa is due to past exposure to a broad range of T_H1 and T_H2 inducing pathogens, subclinical co-infections that are difficult to detect and diagnose, or to other host and environmental factors remains to be determined.

In addition to an up-regulation of soluble activation markers (TNF-α, IL-6, CCL2), a marked increase in the frequency of CD14$^+$D16$^+$ monocytes was observed in HIV-1$^+$ compared to uninfected Africans, and in African populations vs. their non-African counterparts (54). These findings are consistent with previous studies describing elevated levels of CD14$^+$CD16$^+$ monocytes in ART-naïve patients with advanced subtype B infection (26, 27) and in HIV-1$^+$ Malawian women with malaria (38). In the Malawian study, CD14$^+$CD16$^+$ monocytes were also found to express higher levels of CCR5 than their CD16$^-$ counterparts and were significantly more likely to harbor HIV-1 (38). The factors driving the expansion of CD14$^+$CD16$^+$ monocytes and the clinical relevance of this expansion are not well defined.

Perhaps, the most compelling evidence that monocytes play a role in clinical disease is derived from studies showing that $CD14^+CD16^+$ monocytes infiltrate into the CNS where they differentiate into perivascular macrophages and promote tissue injury (32, 55, 56). A recent study, conducted in ART-experienced patients, reported that high levels of circulating LPS were positively correlated with both monocyte activation (as defined by high levels of CD69 and HLA-DR expression) and HIV-1-associated dementia (HAD) (35). Based on these findings, it was suggested that LPS may contribute to the pathology of HAD by promoting increased activation and trafficking of $CD14^+CD16^+$ monocytes into the brain, altering the permeability of the blood-brain barrier (57, 58). Interestingly, similar to my study, only a select subset of monocyte activation markers were positively correlated with LPS, namely sCD14, CD69 and HLA-DR. No positive correlations were detected between LPS and CCL2, or between LPS and the frequency of $CD14^+CD16^+$ monocytes, suggesting that factors other than LPS were driving this activation profile (35).

My data showing that HIV-1 viremia is positively correlated with both $CD14^+CD16^+$ monocytes and plasma levels of CCL2, together with results obtained in the pre-HAART era, suggests that HIV-1 rather than LPS may be the primary factor driving the expansion of $CD14^+CD16^+$ monocytes and the upregulation of CCL2, at least in ART-naïve patients. This view is supported by a recent study of differential gene expression in monocytes from patients with high vs. low viremia (59). In this study, patients with high levels of plasma viremia had an increased frequency of monocytes with an "invasive chronic inflammatory" phenotype characterized by elevated levels of CD16, CCL2, CCR5 and CD169 mRNA (59). Patients with HAD also had increased levels of CD69 mRNA, an activation marker associated with apoptosis and cell death (27, 60). In this regard, previous studies conducted in our laboratory (61, 62) have detected increased levels of CCL2 in the cerebral spinal fluid of European AIDS patients with either HIV-1- or CMV-encephalitis. In addition, it has also been shown that HIV-1 replication and exposure to viral proteins, namely Tat and gp120Env, could drive the upregulation of CCL2 in MDM (63, 64).

Furthermore, CCL2 has been shown to upregulate virus replication in PBMC isolated from HIV-1 infected patients (65) and a polymorphism in the CCL2 promoter has been linked to increased production of this chemokine, higher levels of HIV-1 viremia and HAD (36).

In addition to CCL2 and CD16, we detected increased levels of circulating CXCL10 in African AIDS patients without OIs. Like CCL2, CXCL10 has been implicated in the development of HIV-1-associated neuropathy (36, 66) whereas, *in vitro*, this chemokine has been shown to promote viral replication (67). While both chemokines are produced by $CD14^+CD16^+$ monocytes, CXCL10 is typically secreted in response to IFN-γ, promotes cell recruitment, adhesion to endothelial cells and induces neuronal apoptosis (68, 69). Consistent with previous studies (70, 71), levels of CCR5-binding chemokines were either unchanged (CCL3 and CCL4) or decreased (CCL5) in $HIV\text{-}1^+$ vs uninfected Africans. This observation, together with an independent report describing the up-regulation of CCR5 in $HIV\text{-}1^+$ Africans (47) suggests that the high prevalence of R5-using HIV-1 strains in Africa may be driven by two reciprocal mechanisms: an upregulation of CCR5 and a down-regulation of the CCR5 ligand, CCL5/RANTES.

Although my results support a link between microbial translocation and LPS-induced monocyte activation, this relationship appears to be restricted to the differential upregulation of a distinct subset of immune modulators that include TNF-α and sCD14. Additionally, my results support the existence of a clear link between HIV-1 viremia, the expansion of $CD14^+CD16^+$ monocytes and the production of CCL2, a chemokine that plays a key role in the recruitment and trafficking of monocytes. Additional studies are needed to determine the relative contribution of these distinct but interrelated activation profiles to the pathogenesis of HIV-1/AIDS and their potential differential response to the introduction of ART.

5.4 TABLES

TABLE 1. Demographic and clinical characteristics.

Gender	Male Female	n=8 N=11
Age (years)	Mean ± S.D. Median (range)	35 ± 9.3 33 (24-58)
Log plasma HIV RNA (copies/ml)	Mean ± S.D. Median (range)	4.5 ± 1.14 4.6 (1.7-6.1)
CD4 T-cell count (cells/µl)	Mean ± S.D. Median (range)	108 ± 97 89 (6-378)

TABLE 2. Cytokine activation profiles.

	HIV+ (n=19)	HIV- controls (n=10)	p value (HIV+ vs. CONTROL)	Published means HIV+	Published means HIV-	Reference
TH1/TH2 Cytokines (pg/ml)						
IFN-gamma	591 (72-2448)	130 (24-272)	0.006	102 (0-1120)	1092 (0-4915)	54
IL-4	4.8 (0.9-22.9)	1.4 (0.5-2.6)	0.007	1 (0-6)	0 (0-279)	54
M1/M2 Cytokines (pg/ml)						
IL-12	20 (3.9-83)	8 (2.8-26)	0.006	2.3 (0.6-4.5)	8.6 (4.2-19.8)	53
IL-10	40 (3.7-213)	11 (0.6-31)	0.012	12 (2-22)	4 (1-76)	54
Chemokines (pg/ml)						
CCL3	51 (0-129)	36 (0-76)	n.s.	22 (S.D. 5)	32.4 (S.D. 2.6)	70
CCL4	138 (48-347)	179 (100-360)	n.s.	46.9 (S.D. 6.9)	77.9 (S.D. 10)	70
CCL5	2896 (582-21985)	3441 (1034-5396)	0.0096	612 (S.D. 125)	1538 (S.D. 106)	70
CXCL10	2969 (690-19228)	1691 (285-10228)	0.02	1300 (5-1400)	50 (0-500)	66

TABLE 3. Monocyte activation profiles

	HIV+ (n=19)	HIV- controls (n=10)	p-value (HIV+ vs. CONTROL)	Published means HIV+	Published means HIV-	Reference
CD14+CD16+ Mo (%)	26 (7-38)	13 (7-20)	0.002	16	6.5	27
sCD14 (ug/ml)	2.6 (1.8-3.8)	1.1 (0.3-2)	<0.001	2.5	1.8	19
TNF-alpha (pg/ml)	160 (42-677)	60 (20-109)	0.002	13	0 (0-14)	54
IL-6 (pg/ml)	26 (8-116)	11 (5-18)	0.03	11	1 (0-9)	54
CCL2 (pg/ml)	141 (17-284)	36 (10-61)	0.0003	59	49	60

5.5 FIGURE LEGENDS

Figure 5.1. Disproportionate increase in IL-10 relative to IL-12 in HIV-1-infected vs. uninfected African controls. Levels of IL-4, IFN-γ, IL-10 and IL-12 were quantified in the plasma of treatment-naïve AIDS patients (n =19) and healthy uninfected controls subjects. The ratios of IL-4:IFN-γ and IL-10:IL-12 were calculated to determine whether HIV-1 infection was associated with a shift in the balance between type 1 (IFN-γ, IL-12) and 2 (IL-4, IL-10) cytokines.

Figure 5.2. Increased frequency of circulating $CD14^+CD16^+$ monocytes in African AIDS patients with and without OIs. The frequency of $CD14^+CD16^+$ monocytes within the total monocyte population was analyzed in treatment-naïve patients with no evidence of active opportunistic infections (OI) including tuberculosis and enteric parasites (n = 19); in patients with a range of different bacterial (n = 10), viral (n = 9) and fungal (n = 7) OIs and in uninfected controls (n = 10). Total and $CD14^+CD16^+$ monocytes were distinguished from granulocytes and NK cells by their forward and side scatter characteristics and their expression CD14 (PE-Cy5) and CD16 (FITC).

Figure 5.3. Increased levels of monocyte activation in $HIV\text{-}1^+$ compared to healthy uninfected Africans. Levels of sCD14, TNF-α, IL-6 and CCL2 were quantified in the plasma of untreated AIDS patients with no evidence of opportunistic or enteric co-infections (n = 19) and in uninfected African controls (n = 10).

Figure 5.4. Up-regulation of CCL2 is associated with an increased frequency of circulating $CD14^+CD16^+$ monocytes. Spearman correlations (r and p-values) were calculated to determine the relationships between the frequency of circulating $CD14^+$(PE-CY5)$CD16^+$(FITC) monocytes and other markers of monocyte activation in ART-naïve AIDS patients without OIs (n = 19).

Figure 5.5. Increased $CD14^+CD16^+$ monocyte frequency and the up-regulation of CCL2 in African AIDS patients are associated with high HIV-1 viral loads. The

frequency of $CD14^+$(PE-CY5)$CD16^+$(FITC) monocytes and plasma levels of HIV-1 RNA, sCD14, TNF-α, IL-6 and CCL2 were quantified in treatment-naïve African AIDS patients (n = 19). Spearman correlations (r and p-values) were calculated to determine the relationship between HIV-1 RNA levels and markers of monocyte activation.

Figure 5.6. Up-regulation of sCD14 and TNF-α in African AIDS patients are associated with high levels of plasma LPS. **(A)** LPS was quantified in the plasma of HIV negative controls (n = 10) and treatment-naïve African AIDS patients (n = 19). P values were calculated by the Mann-Whitney U test. **(B)** Levels of circulating $CD14^+$(PE-CY5)$CD16^+$(FITC) monocytes, sCD14, TNF-α, IL-6 CCL2 and LPS were quantified in the plasma of treatment-naïve African AIDS patients (n = 19). Spearman correlations (r and p-values) were calculated to determine the relationship between LPS levels and markers of monocyte activation.

5.6 FIGURES

FIGURE 5.1

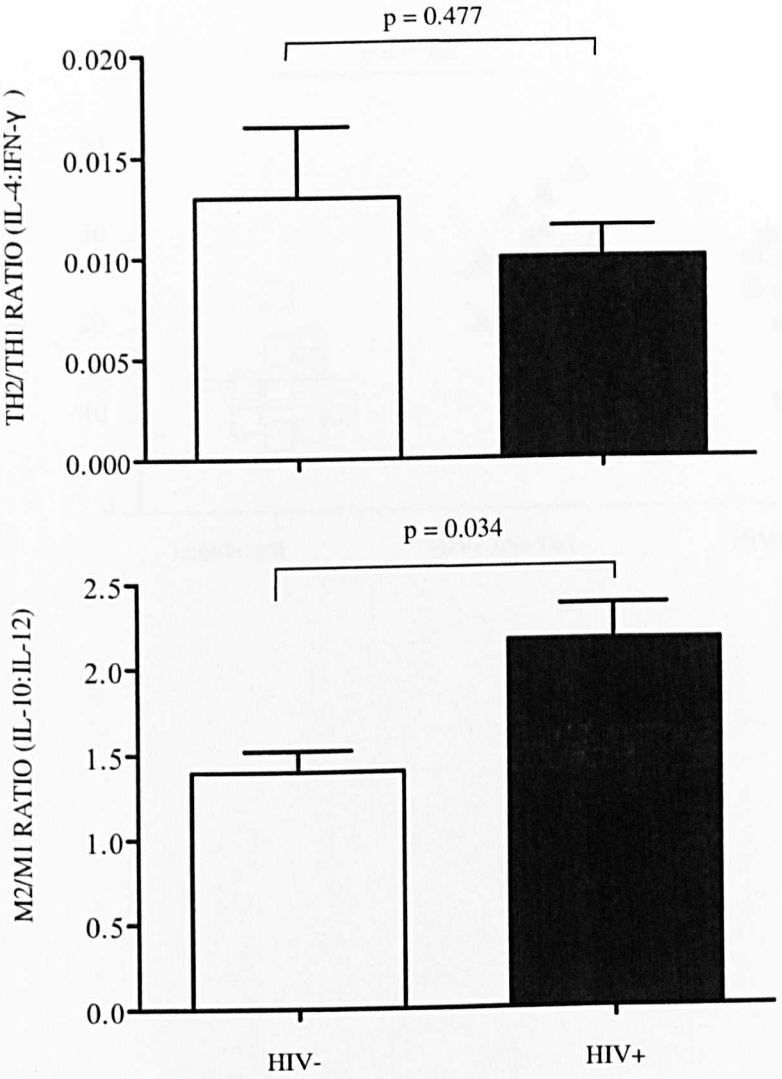


FIGURE 5.2

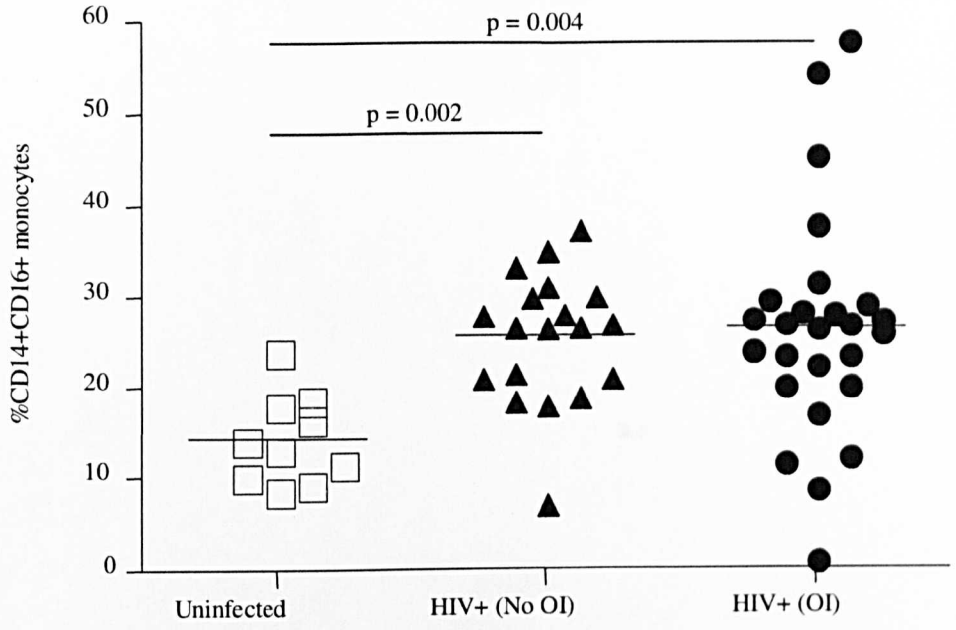

p = 0.004
p = 0.002
%CD14+CD16+ monocytes
60
50
40
30
20
10
0
Uninfected
HIV+ (No OI)
HIV+ (OI)

FIGURE 5.3

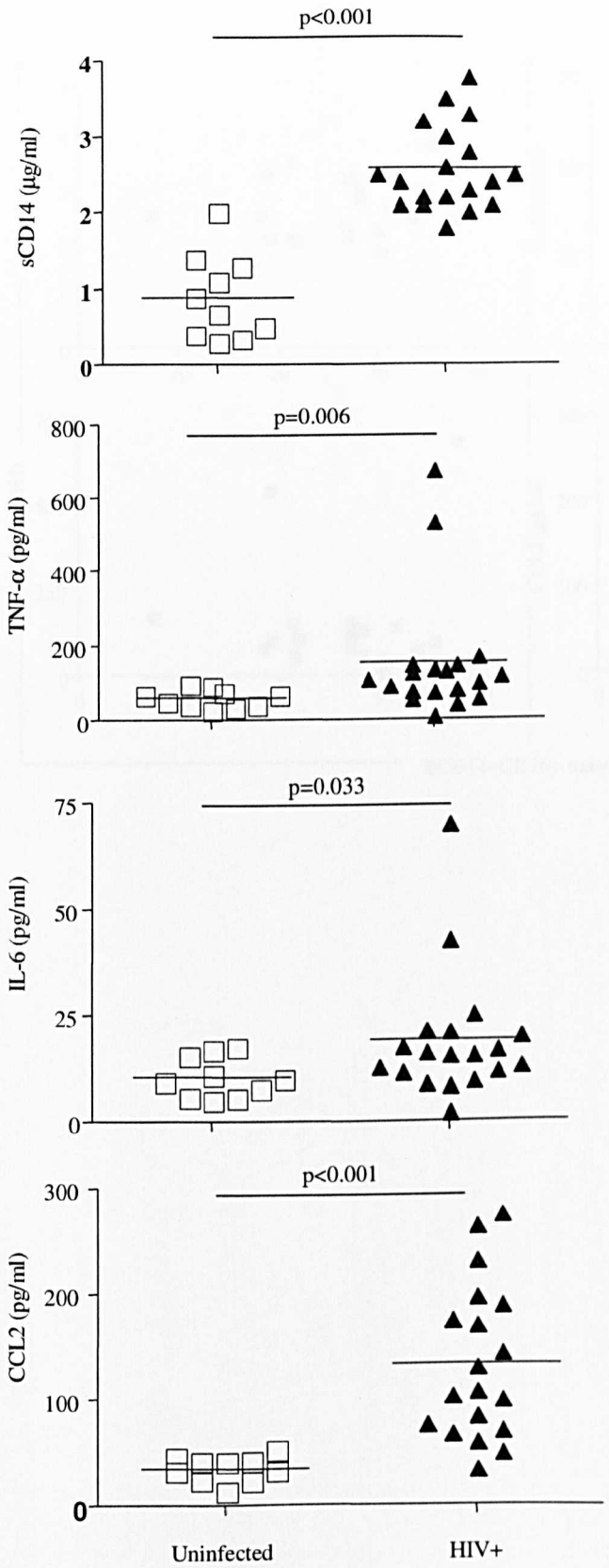

p<0.001
sCD14 (µg/ml)
4
3
2
1
0
p=0.006
TNF-α (pg/ml)
800
600
400
200
0
p=0.033
IL-6 (pg/ml)
75
50
25
0
p<0.001
CCL2 (pg/ml)
300
200
100
0
Uninfected
HIV+

FIGURE 5.4

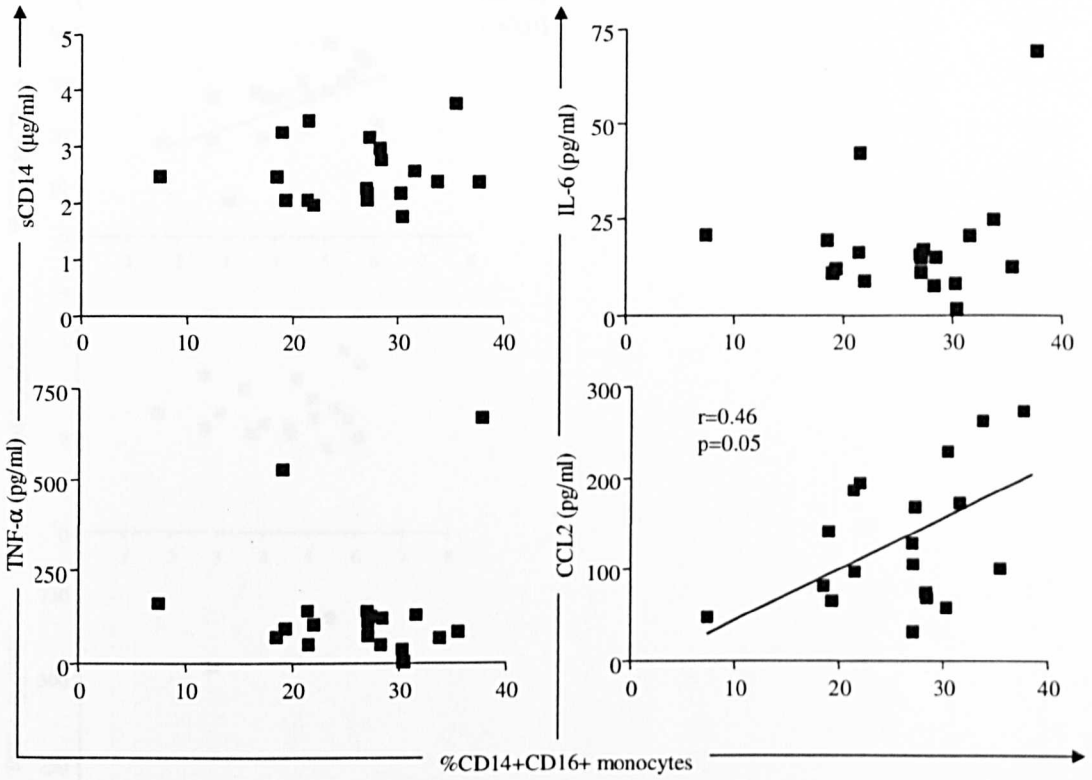
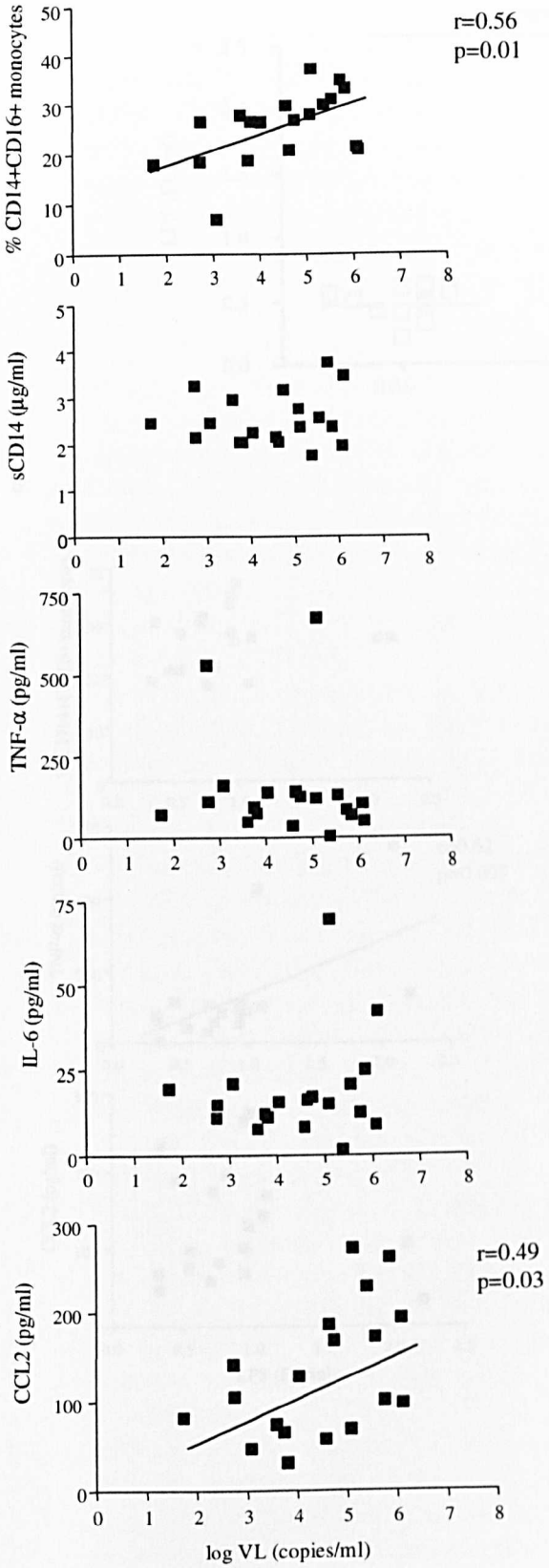
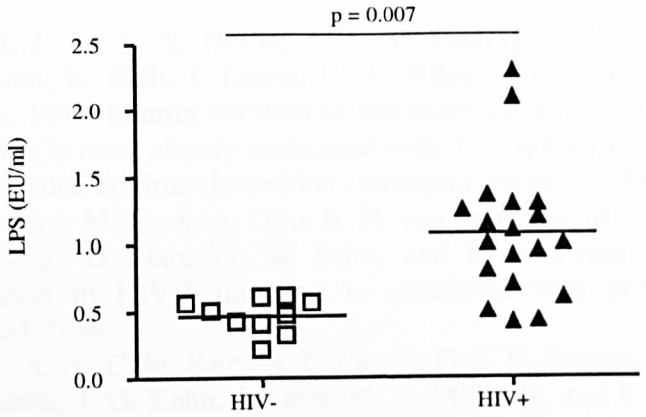

sCD14 (µg/ml)
IL-6 (pg/ml)
TNF-α (pg/ml)
CCL2 (pg/ml)
r=0.46
p=0.05
%CD14+CD16+ monocytes

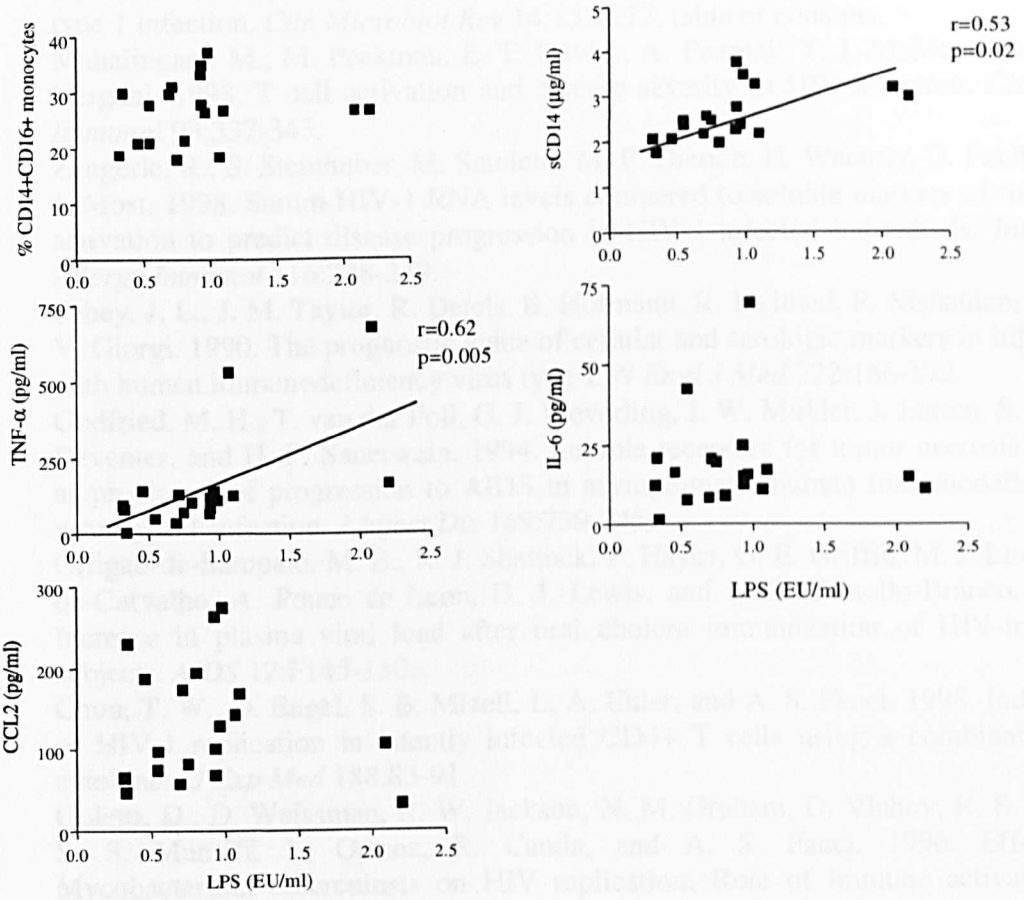
FIGURE 5.5


% CD14+CD16+ monocytes
r=0.56
p=0.01
sCD14 (µg/ml)
TNF-α (pg/ml)
IL-6 (pg/ml)
CCL2 (pg/ml)
r=0.49
p=0.03
log VL (copies/ml)

A.


p = 0.007
LPS (EU/ml)
2.5
2.0
1.5
1.0
0.5
0.0
HIV-
HIV+

B.


% CD14+CD16+ monocytes
sCD14 (µg/ml)
r=0.53
p=0.02
TNF-α (pg/ml)
r=0.62
p=0.005
IL-6 (pg/ml)
CCL2 (pg/ml)
LPS (EU/ml)
LPS (EU/ml)

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CHAPTER 6
PIVOTAL ROLE OF INTESTINAL MACROPHAGES IN CONTROLLING HIV-1 REPLICATION AND FUELING MICROBIAL TRANSLOCATION

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6.0 INTRODUCTION

The gastrointestinal tract (GIT) is major site of HIV-1 pathogenesis (1-3). In contrast to the slow depletion of $CD4^+$ T cells in the peripheral circulation, up to 90% of the $CD4^+CCR5^+$ memory T cells in the gastrointestinal-associated lymphoid tissue (GALT) are lost during the first few weeks of HIV-1 infection (4, 5). Although the exact mechanisms are not known, it has been suggested that this early damage to the GALT may set the stage for increased microbial translocation, chronic activation and progressive immune dysfunction leading ultimately, to the development of AIDS (3, 5-7). However, studies of non-pathogenic SIV infection in animal models have shown that severe $CD4^+$ T cell depletion in the GIT, and at other mucosal sites, is not sufficient to cause high levels of sustained microbial translocation, chronic immune activation or AIDS (8-10). Moreover, in individuals with inflammatory bowel disease (IBD), microbial translocation commonly occurs in the absence of $CD4^+$ T cell depletion (11). Collectively, these data suggest that microbial translocation and progression to AIDS is likely to be a complex phenomenon involving multiple interrelated pathogenic events and immune cell populations.

Although the intestine contains the largest population of macrophages in the body, these cells have received little attention in the setting of HIV-1/AIDS, presumably because of their resistance to HIV-1 infection and their profound "inflammatory anergy" in healthy mucosa (12, 13). Resident macrophages in non-inflamed intestine do not express innate response receptors including CD14 and thus, do not produce inflammatory cytokines in response to microbial stimulation (13-15). In addition, healthy intestinal macrophages do not express co-stimulatory molecules involved in T cell activation (14-16). They do, however, retain an avid ability to phagocytose and kill invading pathogens (14, 15). These unique properties allow for the effective elimination of dangerous pathogens while, at the same time,

protecting the GIT from excessive immune activation due to constant exposure to high levels of commensal bacteria (16).

In this study, we investigated the effects of HIV-1 infection and microbial translocation on the activation of intestinal macrophages and the potential of these cells to contribute to HIV-1 pathogenesis. I detected a marked increase in macrophages expressing innate response (CD14, CD16) and co-stimulatory (CD80, CD86) receptors in both the small and large intestine of HIV-1-infected patients with advanced disease and no evidence of enteric co-infections. In the colon, but not the duodenum, the frequency of CD14+ macrophages was negatively correlated with tissue HIV-1 RNA levels and positively correlated with plasma LPS. I conclude that, similar to IBD, HIV-1 infection is associated with a significant increase in the frequency of activated "inflammatory-like" macrophages and that, in the colon, these cells may play a pivotal role in both the control of HIV-1 replication and the paradoxical enhancement of bacterial translocation and immune activation.

6.1 MATERIAL AND METHODS

6.1.1 *Study participants.* HIV-1-infected South African patients with peripheral blood $CD4^+$ T cell counts of <250 cells/μl and chronic diarrhea were identified at the Comprehensive Care, Management and Treatment (CCMT) Clinic of the Tshwane District Hospital in Pretoria, South Africa. Baseline CD4 counts, HIV-1 plasma viral loads, full blood counts, alanine aminotransferase (ALT) and aspartate amino-transferase (AST) testing as well as stool microscopy and culture were conducted as part of their routine clinical assessment. Patients were considered for the study if they were older than 18 years of age, were ART-naïve, had a weight loss of more than 10% of their body weight or had chronic diarrhea and tested negative for TB in sputum smears stained for acid fast bacilli (AFB). Bacterial and parasitic infections of the intestine were excluded by histological examination of haematoxylin and eosin (H &E) stained tissue sections supplemented with periodic acid-Schiff and Ziehl-Neelsen staining. Cytomegalovirus (CMV) was excluded by immunohistochemistry (DakCytomation, Glostrup, Denmark; clones CCH2 and DDG9). Patients that had no evidence of active bacterial or opportunistic infections (OIs) and had not taken antibiotics for 6 months prior to sampling were considered eligible for recruitment into the study. A total of 18 African patients (10 female, 8 male) with stage III and IV disease (WHO classification) were recruited into the study. As summarized in Table I, these patients had a mean age of 34.9 years (SD ± 10.5), a mean baseline CD4+ T cell count of 106 cells/μl (SD ± 96) and a mean baseline viral load of 4.93 $\log_{10}$ HIV-1 RNA copies/ml of plasma (SD ± 1.0) (**Table 1**). Following clinical assessment, patients were enrolled into a standard NRTI/NNRTI-based treatment program consisting of lamividine (3TC) and stavudine (d4T) in combination with either nevirapine (NVP)(n=7) or effavirenz (EFV)(n=11). Four healthy HIV-1-negative volunteers were recruited from an outpatient clinic. The protocol was approved by the Ethics Committee of the University of Pretoria and each participant gave written informed consent.

6.1.2 *Sampling protocol.* Blood was collected in EDTA tubes at baseline and at 3 and 6 months after the commencement of ART. After obtaining informed consent, biopsies of the duodenum and left colon were obtained at baseline as part of a double balloon enteroscopy using a Fujinon enteroscope and a Radial Jaw 3 biopsy forceps (Boston Scientific). All endoscopic abnormalities were recorded. To examine the effect of ART on mucosal cell populations, post-treatment follow-up biopsies from the duodenum were collected at month 3, and from both duodenum and left colon at month 6. For all procedures, 8-10 biopsies/site were collected for flow cytometry and immediately placed in ice-cold RPMI containing 10% heat inactivated fetal calf serum (FCS). Biopsies collected for the assessment of HIV-1 RNA were snap frozen in liquid nitrogen and stored at -80°C until extraction. Tissue samples for histology were placed in a 10% neutral buffered formalin solution, processed and embedded in paraffin wax within 24 hours of sampling.

6.1.3 *Isolation of mucosal mononuclear cells.* Duodenal and colonic biopsies were washed once in RPMI medium containing 10% FCS and transferred to a 15 ml centrifuge tube containing RPMI + 10% FCS and 0.5 mg/ml collagenase type IV (from *Clostridium histolyticum,* Sigma Aldrich, St. Louis, MO). Samples were incubated at 37°C for 30 minutes in a shaking water bath for optimal dissociation. After incubation in collagenase, cell suspensions were passed through a 70 micron filter (Becton Dickenson Labware, Lincoln Park, NJ) and stored on ice. Remaining tissue fragments were re-digested as described above. The combined single cell suspension was washed to remove any residual collagenase before processing for flow cytometry.

6.1.4 *Monoclonal Antibodies.* The monoclonal antibodies (mAb) used in this study were obtained from a number of sources. Anti-CD45 PE (clone J.33), anti-CD3 PE-Cy5 (clone UCHT1), anti-CD4 FITC (clone 13B8.2) and anti-CD16 FITC (clone 3G8) were purchased from Beckman Coulter (Beckman Coulter, Fullerton, California); anti-CD33 APC (clone 6C5/2), anti-CD80 FITC (clone 37711) and anti-DC-SIGN PE (clone 120507)

were obtained from R&D Systems; anti-CD14 PE-Cy7 (clone 61D3) and anti-CD86 PE (clone IT2.2) was purchased from eBioscience (San Diego, California).

6.1.5 *Quantification of mucosal mononuclear cell subsets using flow cytometry.* Single cell suspensions of biopsies from the duodenum and colon were prepared as described above and subjected to flow cytometry. Briefly, 50-100 µl aliquots of a mucosal mononuclear cell suspension were transferred to individual flow tube (Beckman coulter) and labeled with a specific combination of monoclonal antibodies for 30 minutes at 4°C. Red blood cells were lysed and mononuclear cells were fixed using a TQ-prep instrument and Immunoprep reagents (Beckman Coulter). Macrophage populations were identified based on their forward and side scatter characteristics, as well as their expression of CD33. All CD33 positive cells were CD45 and CD68 positive. Lymphocytes were also gated based on their forward scatter and side scatter properties since back-gating with CD3, CD4 and CD45 mAbs revealed a uniform population of $CD4^+$ lymphocytes in all patients. Samples were analyzed on an FC500 flow cytometer (Beckman Coulter) and a minimum of 50,000 events were acquired per sample.

6.1.6 *Histochemical analysis of macrophage distribution.* Macrophage staining was performed as previously described (17). Briefly, sections of paraffin embedded tissue were pretreated in a microwave oven with two cycles of 5 minutes at 780W in 0.01M citrate buffer and incubated for 2 hours with anti-CD68 antibody (PGM1, 1:500; Dako, Copenhagen, Denmark). Reactions were visualized with a nonbiotin peroxidase detection system (30 minute incubation) using 3,3' diaminobenzidine free base as chromogen (brown staining).

6.1.7 *Measurement of tissue viral loads.* Tissue biopsies (4-12 mg) were manually minced with a razor blade, placed in lysis buffer (BioMerieux) and agitated overnight at RT. The next day RNA was extracted using a guanidinium-silica method (ie. the Magnetic Extraction Reagent Kit from BioMerieux Inc) with no modifications to the protocol. HIV-1 RNA levels were measured using the Nuclisens Easy Q HIV-1 v.1.2 kit, also with no

modifications to the protocol. Results are reported as number of HIV-1 RNA copies per milligram of tissue, or per ml of plasma.

6.1.8 *Quantification of plasma LPS.* Plasma was isolated from EDTA blood tubes by spinning at 1600 rpm for 10 minutes. After separation, plasma was stored in 0.5 ml aliquots at -80°C until needed for testing. Plasma LPS levels were quantified using the Limulus Amebocyte Lysate (LAL) assay QCL-1000 (Lonza, Valais Switzerland). Samples were diluted 1:3 with LAL Reagent Water, inactivated for 30 minutes at 65°C and tested in a 96 well microplate according to manufacture's instructions.

6.1.9 *Statistical analysis.* Prism 5 from GraphPad Software (La Jolla, CA) was used for statistical analyses. Paired observations were compared using Wilcoxon matched pair tests and the Mann-Whitney U test was used to compare means between groups. Linear correlations were assessed using Spearman's rank correlation coefficient. Two-tailed P values less than 0.05 was considered significant.

6.2 RESULTS

6.2.1 *$CD4^+$ T cell depletion is less severe in the colon and correlates with higher levels of tissue HIV-1 RNA and slower immune restoration.* The depletion of $CD4^+$ T cells in the duodenum of ART-naïve patients with advanced HIV-1 disease was more severe than that observed in the colon (mean of 3.3±2.6% vs. 10.6±5.7%, respectively; $p < 0.0001$) (**Figure 6.1A**). Following the initiation of ART, there was a modest but statistically significant increase (from 3.3±2.6 % to 5.0±2.7%; p=0.0002) in $CD4^+$ T cells in the duodenum, reaching peak levels at month 3. This early increase in $CD4^+$ T cells in the duodenum, but not in the colon, is consistent with studies showing a more rapid immune recovery in patients with low vs. high peripheral blood $CD4^+$ T cell counts (18-20). Our data suggest that a similar phenomenon may occur in severely immunodepleted tissues. Although viral RNA was detected in all patients, pre-treatment levels of HIV-1 RNA were higher in the colon than in the duodenum (4.57 vs. 4.06 $\log_{10}$ copies/mg tissue; p=0.01), a finding that is consistent with the higher proportion of $CD4^+$ target cells in this tissue. The initiation of ART led to a marked reduction in HIV-1 RNA at both sites (**Figure 6.1B**). Although there was no correlation between $CD4^+$ T cells and viral RNA levels at baseline, in either the duodenum or the colon (**Figure 6.1C**), the peak increase in $CD4^+$ T cells in the duodenum at month 3 corresponded to the nadir in HIV-1 RNA in this tissue (**Figures 6.1A and 6.1B**).

6.2.2 *Differential depletion, restoration and distribution of macrophages in the colon vs duodenum of patients with advanced HIV-1 disease.* To our knowledge there is currently no information on potential differences in the frequency, phenotype and function of macrophages in different anatomical sites of the GIT during HIV-1 infection. Although the patterns were similar, the depletion of intestinal macrophages was less severe than CD4+ T cells with both sites retaining relatively large numbers of cells (**Figure 6.2B**). As noted for $CD4^+$ T cells, the loss of $CD33^+$ macrophages was more pronounced in the duodenum than in the colon (mean of 18.0 ± 6.4% vs 26.6 ± 9.8%, respectively; p=0.018)

(**Figure 6.2A**). Also as observed for CD4+ T cells, the initiation of ART was associated with a significant increase in duodenal, but not colonic macrophages. Macrophage reconstitution occurred rapidly reaching peak levels at month 3 followed by a plateau 6 months after the introduction of ART. Despite this increase, macrophage levels at month 6 (in both the duodenum and colon) were still below those observed in uninfected controls (**Figure 6.2A**).

Immunohistochemical staining with CD68 indicated that, in the majority of biopsy samples, macrophages were concentrated immediately beneath the epithelium covering the luminal surface of the lamina propria. This pattern is commonly encountered in all regions of the GIT including the colon and duodenum (14, 15) and is characteristic of a healthy intestinal mucosa (**Figure 6.2B**). Interestingly, 20% of colon specimens from AIDS patients showed an altered distribution in which the macrophages were diffusely scattered throughout the lamina propria. After the administration of ART, these macrophages assumed a normal sub-epithelial pattern suggesting that the altered distribution was reversible and HIV-1-related (**Figure 6.2C**).

6.2.3 *AIDS is associated with an increased frequency of intestinal macrophages expressing innate response and co-stimulatory molecules.* In sharp contrast to the GIT of healthy individuals (14-16), macrophages in the duodenum and colon of AIDS patients expressed elevated levels of receptors for LPS (CD14) and Fcγ RIII (CD16), as well as increased levels of co-stimulatory molecules (CD80, CD86) involved in the activation of T cells (**Table 2**). These findings are very similar to those reported in the inflamed mucosa of patients with IBD, with approximately one third of intestinal macrophages displaying an altered phenotype (14). Interestingly, the levels of these activation markers were consistently higher in the colon than the duodenum (**Table 2**, i.e. CD14 30.9±11.6 vs 20.2±9.8% respectively, p=0.0029) suggesting that the colonic microenvironment may further contribute to, and drive, this activated phenotype.

In the duodenum, ART was associated with a transient increase (26.9 ± 9.5%) in the frequency of $CD14^+$ macrophages at month 3 followed by a return toward baseline levels (19.6±5.8%) 6 months after the initiation of therapy (**Figure 6.3A**). As previously reported for $CD4^+$ T cells, this increase may be due to a transient redistribution of monocytes during early therapy. Although the colon was not sampled at the 3-month time point, a significant decrease in $CD14^+$ macrophages was observed 6 months after the introduction of ART (ie. from 30.9% at baseline to 19.1% at month 6; p=0.018) (**Figure 6.3A**). Although the colon had higher pre-treatment levels of $CD14^+$ macrophages, both tissues had similar post-treatment (month 6) levels of these cells. However, it is important to note that the frequency of these cells, in both the duodenum and colon, was still substantially higher than that observed for healthy uninfected individuals.

HIV-1 infection had no effect on the expression of DC-SIGN on resident intestinal macrophages (a cell population that lacked expression of CD14, CD16, CD80 and CD86) in either the duodenum or colon, or in biopsies sampled before and after the initiation of ART (Figure 3B). This finding suggests that the increase in macrophages expressing innate response and co-stimulatory molecules is most likely due to an influx of newly recruited inflammatory monocytes rather than alterations in the phenotype of resident macrophages.

Further analysis of the relationships among inflammatory markers revealed strong positive correlations between CD14 and CD16 (approaching significance, r=0.47, p=0.08), CD80 and CD86 expression (**Figure 6.3C**) suggesting that these receptors are tightly co-regulated. Based on these associations and IBD studies showing that CD14 expression strongly correlates with pro-inflammatory cytokine production (23, 24), CD14 was selected as a representative activation marker for further investigations.

6.2.4 *Frequency of $CD14^+$ macrophages in the duodenum correlates with CD4+ T cell levels.* To investigate whether the increased recruitment of inflammatory macrophages to the duodenum occurred in response to HIV-1, and whether this recruitment was associated with increased microbial translocation, we examined the relationships between

circulating plasma LPS and tissue levels of $CD14^+$ macrophages, $CD4^+$ T cells and HIV-1 RNA. The frequency of $CD14^+$ macrophages in the duodenum was weakly correlated with tissue $CD4^+$ T cell levels (P=0.082) (**Figure 6.4**) suggesting that recruitment of activated monocytes may be dependent on T cell help. In contrast, no correlations were detected between CD14+ macrophages in the duodenum and either tissue viral loads or plasma LPS also suggesting that in the absence of adequate $CD4^+$ T cell help, inflammatory macrophages in the duodenum had little impact on either viral replication or microbial translocation.

6.2.5 *Increased frequency of $CD14^+$ macrophages in the colon is positively correlated with bacterial translocation and negatively correlated with tissue HIV-1 RNA.* In contrast, in the colon, a tissue with a more preserved immune environment, no correlation was detected between the frequency of $CD14^+$ macrophages and $CD4^+$ T cells. Instead, we found a strong positive association between $CD14^+$ macrophages and levels of plasma LPS (P=0.002) and a weaker, but statistically significant (P=0.035) negative correlation between $CD14^+$ macrophages and tissue HIV-1 RNA levels (**Figure 6.5**). Thus, in the colon with a more intact immune system and higher levels of CD4+ T cell help, HIV-1-related recruitment of inflammatory macrophages appears to be sufficient to induce a response that is partially effective in controlling viral replication. However, in this setting, the increased frequency of inflammatory macrophages may also produce increased levels of inflammatory cytokines, damaging the epithelium and facilitating microbial translocation and LPS-induced activation.

6.3 DISCUSSION

AIDS in Africa was initially referred to as "slims disease" because of the large number of HIV-1-infected individuals presenting with severe complications of the GIT including diarrhea, inflammation, digestive dysfunction and lymphocyte depletion (25). Recently, advances in understanding mucosal pathogenesis have provided critical new insights into the potential causes of HIV-1 enteropathy and $CD4^+$ T cell depletion and have rekindled interest in the GIT as a major site of pathogenesis (1, 3). These studies have also shown that, although there is a rapid and profound loss of T-helper cell function in the GIT during acute infection, the largest reservoir of T cells in the body (26-28), progression to AIDS is dependent on additional factors related to persistent viral replication, microbial translocation and chronic immune activation (5, 29). However, it is still unclear whether immune activation is a cause or consequence of bacterial translocation and the mechanisms linking these various pathogenic factors to HIV-1 pathogenesis in the GIT have not been fully elucidated.

Here, I report that HIV-1 infection is also associated with changes in the frequency, distribution and phenotype of intestinal macrophages and that these changes are associated with regional differences in the extent of $CD4^+$ T cell depletion. In the small intestine, a region of intestine that is characterized by discrete lymphoid aggregations known as Peyer's patches (16, 28), a near-complete depletion of $CD4^+$ T cells was observed in all patients. Despite this profound loss of $CD4^+$ T cells, moderately high levels of viral replication were still detectable in this tissue. In contrast, in the colon, a site that contains a diffuse distribution of lymphoid aggregates (16, 28), $CD4^+$ T cell depletion was less severe and more variable suggesting that, in our patient cohort, this tissue had a more intact immune system and a higher level of residual $CD4^+$ helper T cell function. Consistent with the high prevalence of $CD4^+$ T target cells, the colon also had higher levels of HIV-1 RNA. The reasons for these clear-cut regional differences are not known, but may be related to the unique structural and organizational properties of the two tissues. The intestine is the largest

and most complex component of immune system and its functionally distinct regions may affect the functional activities of its immune cells (16).

As recently reported for $CD13^+$ myelomonocytic cells in the terminal ileum (30), we found that $CD33^+$ macrophages were significantly depleted in both the duodenum and colon of HIV-1-infected patients. As observed for CD4+ T cells, macrophage depletion was less severe in the colon vs. the duodenum and, in 20% of patients, was associated with an altered distribution. Instead of being concentrated immediately beneath the epithelium(14, 15), the majority of colonic macrophages in these patients were diffusely distributed throughout the lamina propria. Whether this altered pattern is due to a re-distribution of resident sub-epithelial macrophages or an increased influx of newly-recruited monocytes remains to be determined but, as discussed below, it is most likely due to a newly acquired population of activated macrophages. Interestingly, six months after the initiation of antiretroviral therapy, these intestinal macrophages displayed a typical sub-epithelial distribution suggesting that the changes were HIV-1-related.

Following the initiation of ART there was a small but statistically significant increase in both $CD4^+$ T cells and $CD33^+$ macrophages in the duodenum, but not in the colon. Interestingly, the pattern of duodenal reconstitution was similar for both cell types and involved a peak increase at 3 months followed by a small decrease at month 6. This pattern is compatible with that observed in peripheral blood in which an initial redistribution of $CD4^+$ T cells is followed by a more sustained period of "true" immune reconstitution (18-20). The early restoration of these cell populations in the duodenum, where CD4+ T cell depletion was more severe, is consistent with studies showing that patients with low peripheral blood $CD4^+$ T cell counts show a more rapid and dramatic increase in circulating $CD4^+$ T cell levels following the introduction of HAART (18-20). Although the mechanism is not known, it has been suggested that this phenomenon may reflect a more intense homeostatic pressure at the lower limits of CD4+ T cell renewal (18-20). Despite the lack of a baseline correlation between tissue viral loads and the frequency of either CD4+ T cells or CD33+ macrophages,

immune restoration in the duodenum was inversely related to the pattern of HIV-1 RNA clearance in this tissue suggesting that HIV-1 is likely to be a primary determinant driving the depletion of both cell types, either directly or indirectly.

Perhaps the most important finding, however, was that HIV-1 infection is associated with marked increase in the frequency of intestinal macrophages expressing co-stimulatory (CD80, CD86) and innate response receptors for LPS (CD14) and immunoglobulin G (CD16). The increase in these receptors, which was detected in the absence of enteric co-infections, was consistently 2- to 3-fold greater in the colon compared the duodenum. Interestingly, these "inflammatory-like" changes are highly reminiscent of the events occurring during inflammatory bowel disease (IBD) (31-33). In IBD, this dysregulated immune response is independent of $CD4^+$ T cell depletion and is directed against harmless luminal microflora rather than a virus such as HIV-1 (34). Furthermore, IBD pathogenesis is related to defects in antigen presenting cell (APC) function and APC-T cell interactions that result in aberrant T cell responses and ultimately, chronic inflammation (34). Although these interactions are likely to be more complex in the presence of HIV-1, the observed upregulation of co-stimulatory molecules on intestinal macrophages may also drive inflammation and aberrant T cell responses in the GIT of HIV-1-infected individuals. Moreover, the upregulation of $CD14^+$ receptors on these cells may result in increased responsiveness to bacterial products (23). In the context of increased permeability of the epithelial barrier (a process that begins in acute infection) and increased microbial translocation (3, 5, 6), enhanced expression of CD14 suggests a mechanism whereby microbe-mediated activation of intestinal macrophages may fuel chronic inflammation.

In contrast to the up-regulation of innate response and co-stimulatory molecules, there were no significant changes in the frequency of duodenal or colonic macrophages expressing DC-SIGN. Thus, these "resident" $DC\text{-}SIGN^+$ macrophages (which do not express CD14, CD16, CD80 or CD86) (21, 22) do not appear to be affected by either HIV-1 or ART. This finding is consistent with *in vitro* studies showing that resident intestinal macrophages

do not exhibit the same plasticity as other tissue macrophages and cannot be induced to express innate or co-stimulatory molecules (13). Collectively, these data suggest that HIV-1-associated alterations in the phenotype of intestinal macrophages are the result of an increased recruitment of "inflammatory" monocytes rather than a switch from a resident to an activated phenotype. This view has important implications for treatment strategies. By limiting the ongoing recruitment of inflammatory monocytes it may be possible to prevent microbial translocation and reduce inflammation.

To investigate the potential implications of macrophage activation on HIV-1 pathogenesis, I examined the tissue-specific relationships between macrophage activation, tissue viral loads, CD4 T cell depletion and microbial translocation as measured by circulating levels of plasma LPS. In the duodenum, a site of severe immune deficiency, I observed a weak positive correlation between the frequency of $CD4^+$ T cells and $CD14^+$ macrophages suggesting that the recruitment of inflammatory monocytes may be dependent on T cell help. No correlation was observed between $CD14^+$ macrophages and tissue viral loads in the duodenum also suggesting that a minimal level of $CD4^+$ T cell help may be required to mediate a protective response. In contrast, a negative correlation was observed between the frequency of $CD14^+$ macrophages and tissue viral loads in the colon suggesting that, in the presence of sufficient $CD4^+$ T cell help, activated "inflammatory-like" macrophages were able to contribute to the control of HIV-1 infection and replication.

In contrast, I observed a strong positive correlation between the frequency of $CD14^+$ macrophages in the colon and plasma LPS suggesting that activated intestinal macrophages may also play a key role in driving microbial translocation and systemic immune activation. Although microbial translocation may occur in other regions of the GIT, it seems logical that the majority of bacterial products would be derived from colon, a site that contains extremely high levels of commensal bacteria (35, 36). Interestingly, in IBD, both microbial translocation and intestinal barrier permeability are tightly linked to macrophage activation. In particular, increased secretion of IL-12 by activated macrophages has been linked to

elevated levels of IFN-γ production in T cells, a process that leads to increased epithelial permeability and further enhancement of macrophage activation and microbial translocation(15). Similar to IBD, macrophage activation seems to be a key factor driving microbial translocation in HIV-1 infected individuals.

Based on these findings, I hypothesize that intestinal macrophages may serve as the missing link for unraveling the complex interactions between HIV-1 pathogenesis, host immunity and microbial translocation. While macrophage activation may be required to mount an effective immune response against HIV-1 in the acute phase of infection, chronic immune activation may lead to aberrant T cell responses, amplification of localized inflammation and increased microbial translocation. Thus, the key to controlling the chronic manifestations of HIV-1 disease in the gastrointestinal may involve interrupting the continuous recruitment of inflammatory monocytes to sites of tissue pathogenesis. Importantly, in the central nervous system, increased recruitment and differentiation of $CD14^{+}CD16^{+}$ monocytes (a subset of inflammatory monocytes that is upregulated in HIV-1 infection) into perivascular macrophages has been strongly linked to the development of AIDS-associated dementia (37-39). Available data, together with results of the current study, suggest that an improved understanding of monocyte/macrophage activation may lead to new insights for the prevention and treatment of HIV-1-associated pathogenicity.

6.4 TABLES

Table 1: Clinical Characteristics of AIDS patients

BASELINE		
Gender	Male Female	n = 8 n = 10
Age (years)	Mean ± S.D.	34.9 ± 10.5
CD4 count (cells/µl)	Mean ± S.D.	106 ± 96
Viral Load (log copies/ml)	Mean ± S.D.	4.93 ± 1.00
HAART	3TC/D4T/NVP 3TC/D4T/EFZ	n = 6 n = 11
TREATMENT		
CD4 count (cells/µl)	Month 3 Month 6	206 ± 124 218 ± 111
Viral Load (log copies/ml)	Month 3 Month 6	2.49 ± 1.15 <LDL

Table 2: Phenotypic Characterization of Intestinal Macrophages

		DC-SIGN+ (% of CD33+ macrophage)**	CD14+ (% of CD33+ Macrophage)	CD16+ (% of CD33+ macrophage)	CD80+ (% of CD33+ macrophage)	CD86+ (% of CD33+ macrophage)
DUODENUM	HIV-	50.1 ± 6.4	12.6 ± 3.2	6.7 ± 1.7	10.9 ± 2.8	12.2 ± 2.2
	HIV+	59.2 ± 15.6	20.2 ± 9.8	16.1 ± 10.6	24.1 ± 8.8	24.3 ± 10.5
COLON	HIV-	N/A	10.5 ± 3.8*	10.1 ± 3.9*	9.2 ± 4.2*	15.1 ± 7.3*
	HIV+	69.4 ±13.1	30.9 ± 11.7	25.4 ± 12.5	30.1 ± 12.4	25.7 ± 11.7

* PUBLISHED VALUES (REFERENCE 15)

** DC-SIGN+ resident macrophages were defined as the % DC-SIGN+ cells of the CD33+ that lacked the innate response and costimulatory molecules

6.5 FIGURE LEGENDS

FIGURE 6.1. Differences in the extent of HIV-1 RNA clearance and $CD4^+$ T cell restoration in the duodenum vs colon of African AIDS patients. A. $CD4^+$ T cell depletion was more severe in the duodenum and showed significant reconstitution after ART. Results are reported as the mean frequency of $CD4^+$ T cells (± S.D, n=18.) in the lymphocyte gate at baseline, and at month 3 and 6 post-ART. Statistical significance ($p>0.05$) between time points is indicated by an asterisk. **B.** ART was associated with a significant reduction in tissue HIV-1 RNA as early as 3 months after the initiation of treatment. Viral RNA levels were measured using Nuclisens Easy Q HIV-1 v.1.2 kit and results are reported as the number of HIV RNA copies per mg of tissue. Data is presented as the mean viral load of the study cohort (n=18, $*p<0.05$). **C.** Degree of $CD4^+$ T cell depletion does not correlate with tissue HIV-1 RNA levels. $CD4^+$ T cell depletion in the colon and duodenum were compared using the Spearman correlation test.

FIGURE 6.2. HIV-1 infection was associated with macrophage depletion in the duodenum and colon, and with an altered distribution of colonic macrophages. A. Depletion of macrophages was more severe in the duodenum and showed significant reconstitution after ART. The frequency of macrophages was evaluated based on CD33 expression. Results are reported as the mean frequency of $CD33^+$ cells after excluding debris and lymphocytes (n=18, $*p<0.05$). The dotted line illustrates the average frequency of CD33+ cells in HIV-1 negative controls. **B.** In a subset of patients, HIV-1 infection in the colon was associated with an alteration in the distribution of macrophages from a normal sub-epithelial ($HIV\text{-}1^-$ and $HIV\text{-}1^+$ phenotype) to a diffusely scattered pattern (HIV+ phenotype 2). $CD68^+$ macrophages (brown cells) were visualized with a non-biotin peroxidase detection system and 3,3' diaminobenzidine free chromogen (40X original magnification). **C.** Alterations in macrophage distribution were resolved by ART. Results shown are representative of 2 of 10 $HIV\text{-}1^+$ AIDS patients tested at baseline and at month 6 after the initiation of ART.

FIGURE 6.3. HIV-1 infection was associated with increased expression of CD14 on intestinal macrophages. A. ART decreased the HIV-1-induced up-regulation of CD14 macrophages in the colon. The frequencies of inflammatory macrophages were calculated by determining the percentage of $CD33^+$ cells that were $CD14^+$. Results are expressed as the means of all study patients (n=18, *$p<0.05$). **B.** DC-SIGN, a marker expressed on resident macrophages, was not altered by either HIV-1 infection or ART. The data obtained by flow cytometry is expressed as mean value (± S.D.). The frequency of DC-$SIGN^+$ macrophages represents the percentage of $CD33^+$ cells that are also DC-$SIGN^+$. **C.** CD14 expression on macrophages was positively correlated with other inflammatory molecules. The relationship between CD14 expression and co-stimulatory molecules was assessed using Spearman correlation test. Results were considered significant if $p<0.05$. Correlations were considered strong if $r > 0.6$.

FIGURE 6.4. Frequency of $CD14^+$ macrophages in the duodenum was weakly correlated with $CD4^+$ T cell levels. Relationships between $CD14^+$ expression on duodenal macrophages and $CD4^+$ T cell depletion, tissue viral loads and circulating levels of LPS were assessed using Spearman correlation. Results were considered significant if $p<0.05$. Correlations were considered strong if $r > 0.6$.

FIGURE 6.5. In the colon, the frequency of $CD14^+$ macrophages was negatively correlated with tissue levels of HIV-1 RNA and positively correlated with plasma LPS. Relationships between $CD14^+$ expression on colonic macrophages and $CD4^+$ T cell depletion, tissue viral loads and circulating levels of LPS were assessed using the Spearman correlation test. Results were considered significant if $p<0.05$. Correlations were considered strong if $r > 0.6$.

FIGURE 6.1

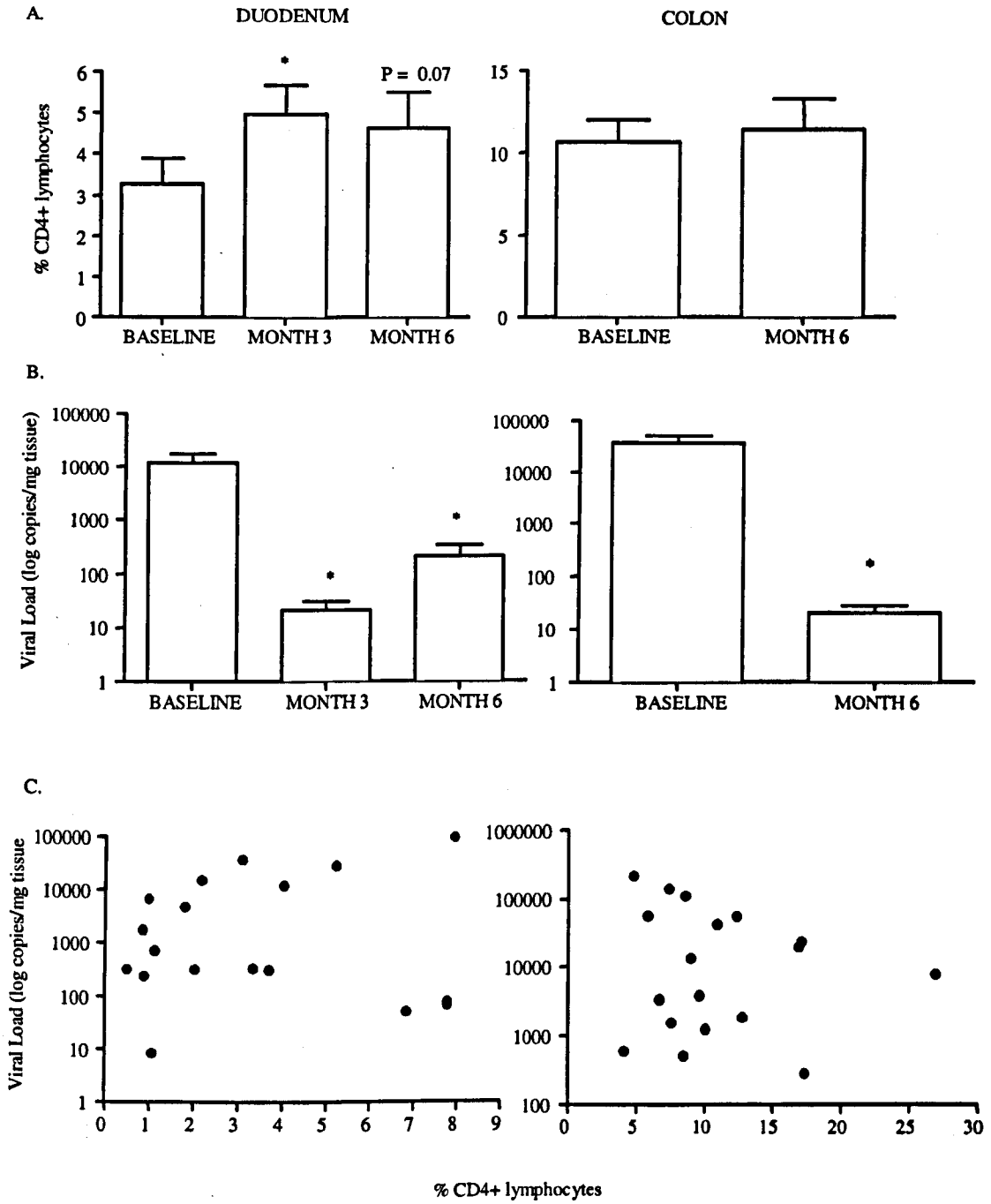
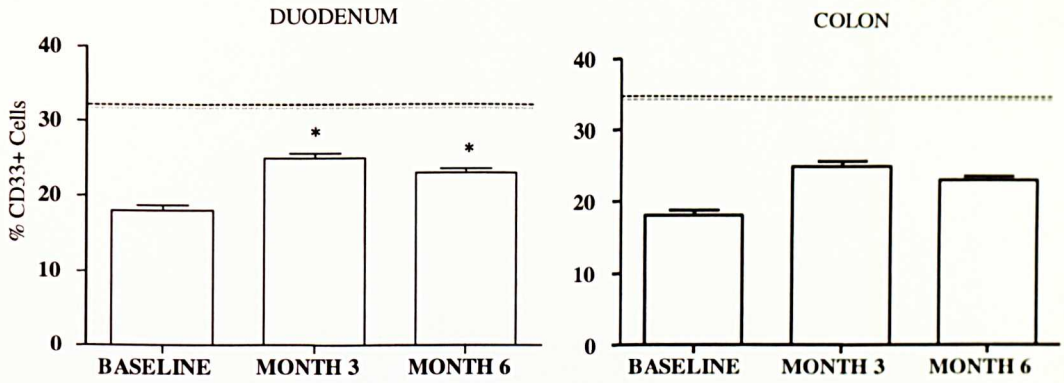
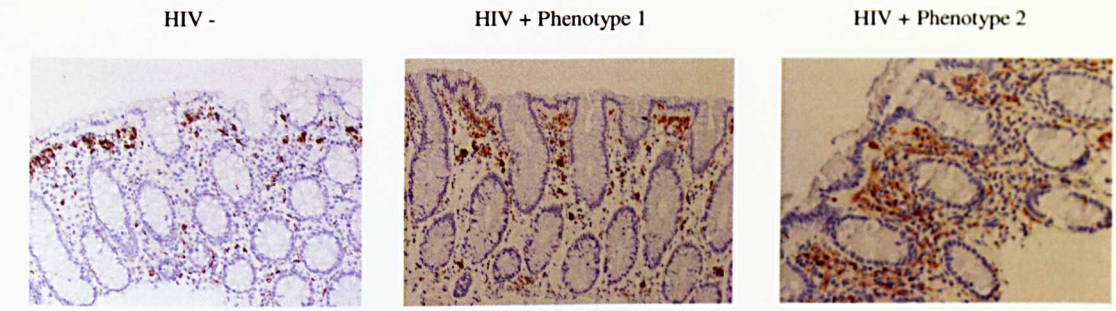

A.
DUODENUM
COLON
% CD4+ lymphocytes
P = 0.07
BASELINE
MONTH 3
MONTH 6
B.
Viral Load (log copies/mg tissue)
C.
Viral Load (log copies/mg tissue
% CD4+ lymphocytes

FIGURE 6.2

A.



B.



C.

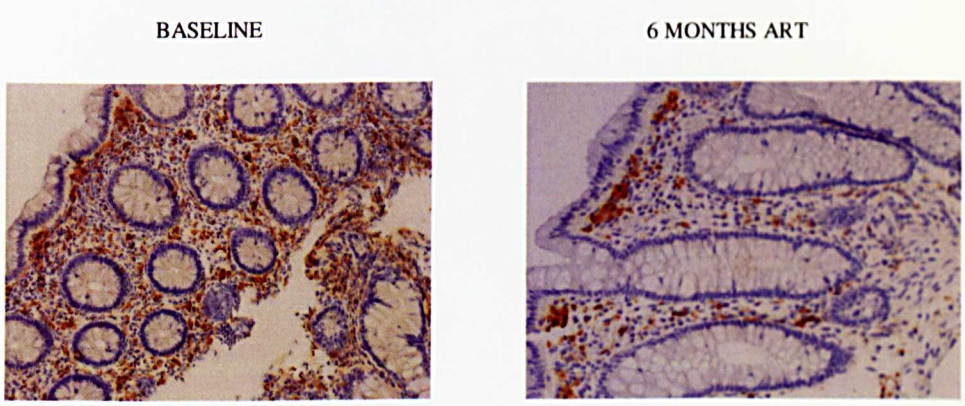
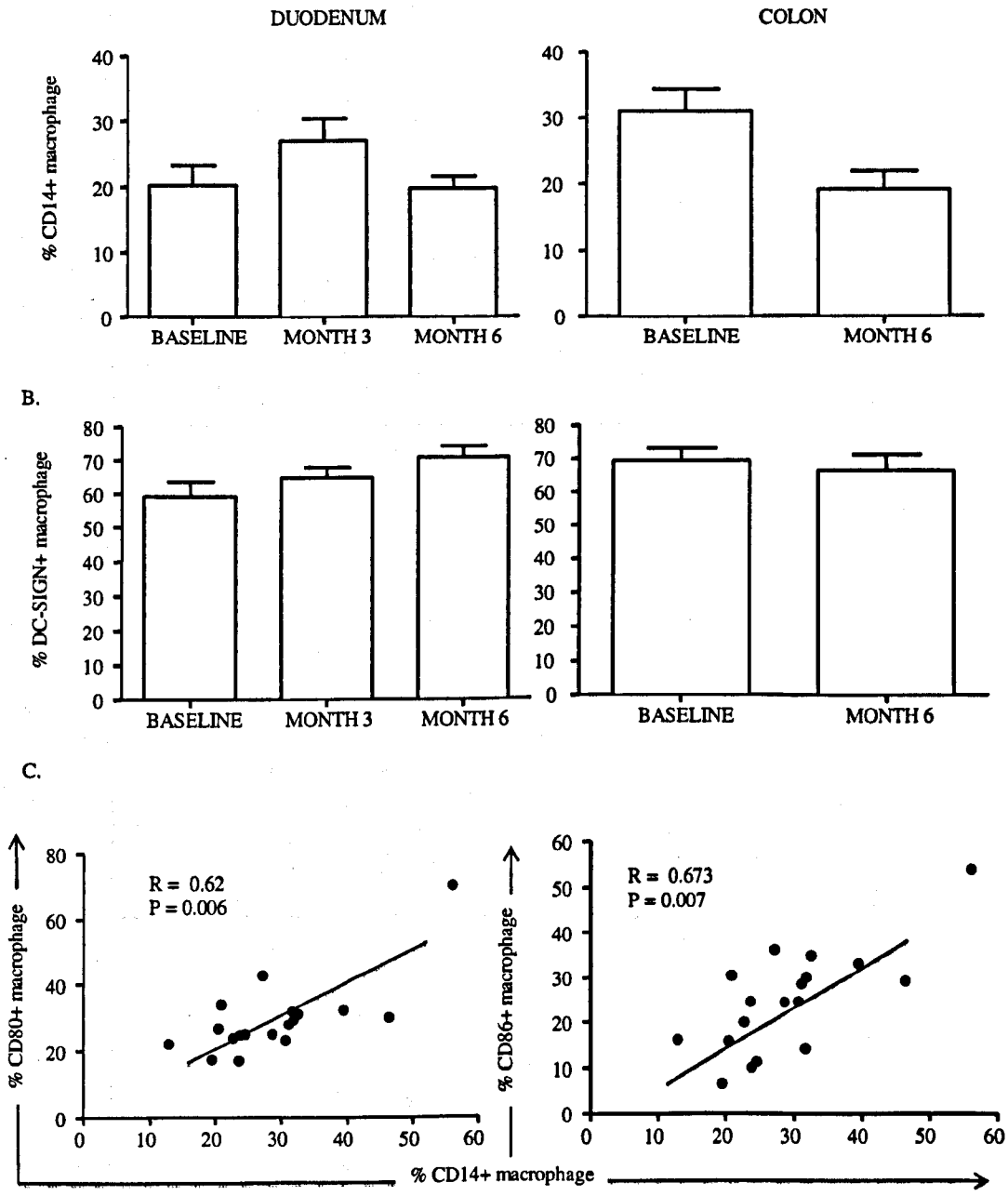
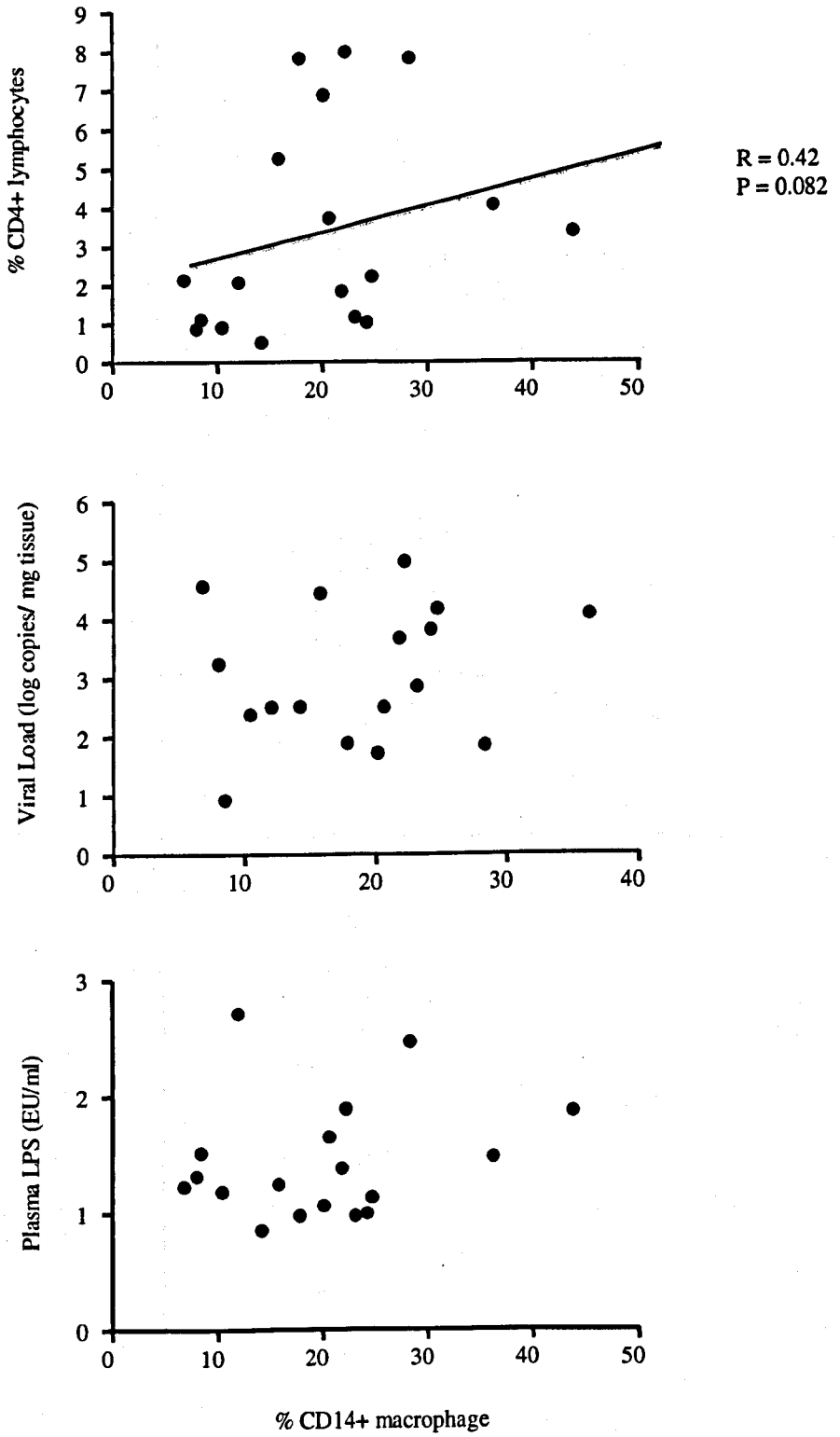
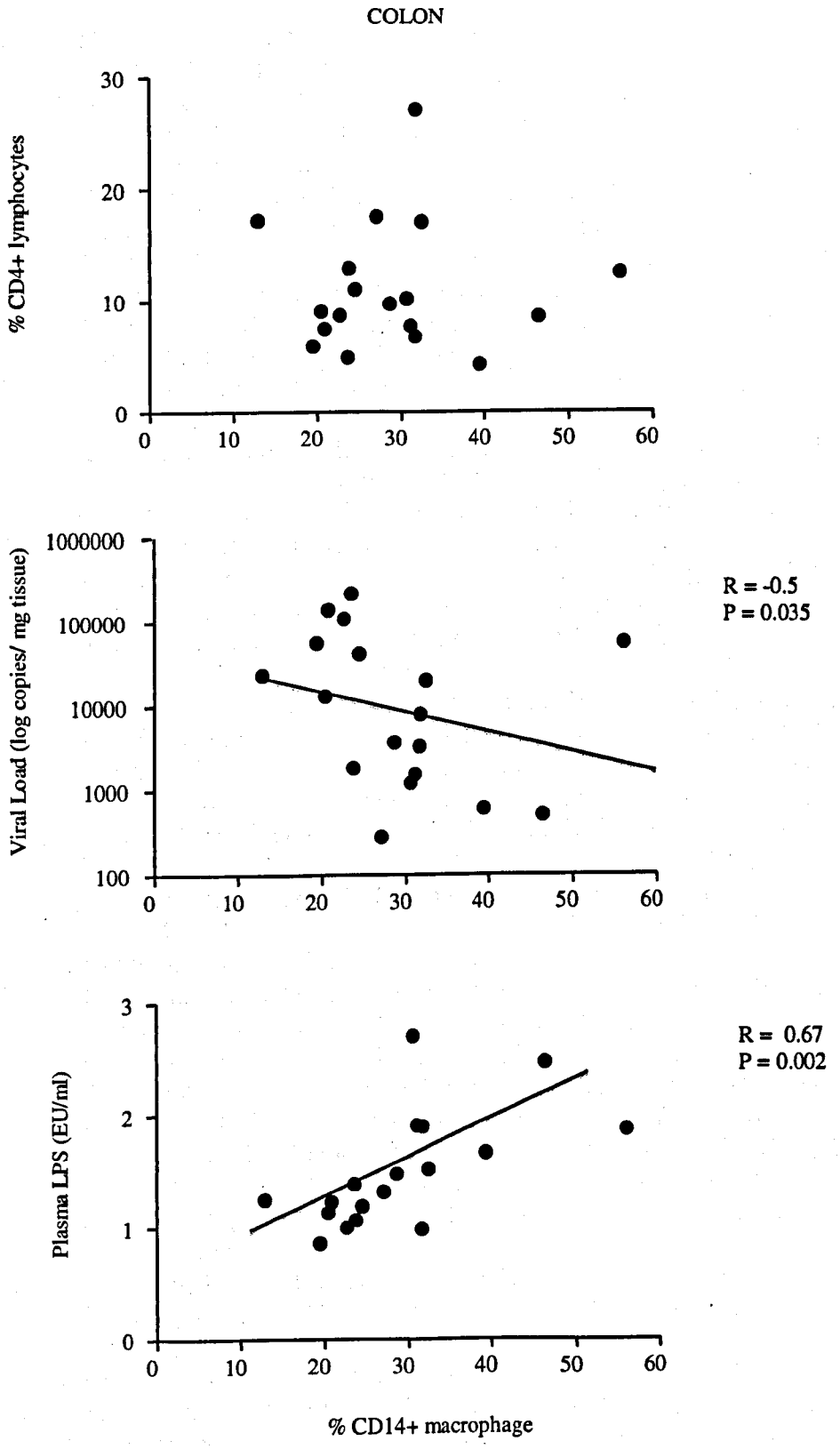


FIGURE 6.3


A.
DUODENUM
COLON
% CD14+ macrophage
0
10
20
30
40
BASELINE
MONTH 3
MONTH 6
B.
% DC-SIGN+ macrophage
0
10
20
30
40
50
60
70
80
C.
% CD80+ macrophage
R = 0.62
P = 0.006
% CD86+ macrophage
R = 0.673
P = 0.007
% CD14+ macrophage

DUODENUM


% CD4+ lymphocytes
R = 0.42
P = 0.082
Viral Load (log copies/ mg tissue)
Plasma LPS (EU/ml)
% CD14+ macrophage


COLON
% CD4+ lymphocytes
30
20
10
0
0
10
20
30
40
50
60
Viral Load (log copies/ mg tissue)
1000000
100000
10000
1000
100
R = -0.5
P = 0.035
Plasma LPS (EU/ml)
3
2
1
0
R = 0.67
P = 0.002
% CD14+ macrophage

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CHAPTER 7
CONCLUSIONS AND PERSPECTIVES FOR IMMUNE BASED THERAPIES

7.0 DISCUSSION

HIV-1 is the most studied pathogen in recorded human history. Although this intense focus on HIV-1/AIDS has led to major advances in the diagnosis and treatment of retroviral infections (ie. the first antiretroviral drugs were developed against HIV-1), there is still no vaccine or therapeutic "cure" for AIDS. Nevertheless, the introduction of HAART has dramatically improved the duration and quality of life for individuals infected with HIV-1. In the US alone, it is estimated that more than 3 million years of life have been saved since the introduction of HAART in 1996 (1). Unfortunately, the cost of these drugs is prohibitive and beyond the reach of many developing countries. Furthermore, prolonged administration of HAART often leads to toxicity, the emergence of drug resistance and ultimately therapeutic failure (2). The relative inability of HAART to completely suppress HIV-1 for prolonged periods of time has been attributed to host, pharmacological and viral factors including the establishment of viral reservoirs (3-5). As discussed below, strategies aimed at suppressing immune activation have the potential to enhance the efficacy of HAART and eliminate HIV-1 in viral reservoirs.

It is becoming increasingly apparent that HIV-1 transmission and pathogenesis are intimately related to the activation state of the host immune system (6-8). Although activation is essential to mount an effective response, it also provides an environment that drives HIV replication and disease progression (9). Increased levels of immune activation correlate with $CD4^+$ T cell depletion (10-13) and, in patients with AIDS, activation is a stronger predictor of progression than plasma viral load (6). In addition to enhancing HIV-1 replication (14-17), chronic immune activation leads eventually to exhaustion and dysfunction of the host immune system (18-21). Recent comparative studies of SIV provided further evidence of the importance of immune activation in mediating SIV pathogenesis (22,23). These studies have shown that AIDS is not an inevitable

consequence of infection and that the main feature distinguishing pathogenic SIV infection in macaques (and HIV-1 in human) from natural (non-pathogenic) infection in sooty mangabeys and chimpanzees is a heightened state of chronic immune activation throughout the course of the disease (22-24). Understanding the factors driving high levels of immune activation is likely to provide new insights into the development of more effective therapeutics.

The above findings offer considerable hope for millions of people in Africa and other regions of the developing world where most individuals only become aware of their infection status during the late stages of disease. The findings suggest that prevention of immune activation during chronic infection may serve to prevent (or delay) the onset of severe immune deficiency and AIDS. Thus, there is an urgent need to determine whether recent SIV data can be directly extrapolated to HIV-1 and whether the suppression (or elimination) of systemic immune activation, when used in conjunction with antiretroviral therapy, can prevent AIDS. It is of note that previous immune-based treatment strategies, including various protocols of structured treatment interruption (STI), focused on activating rather than suppressing the host immune system (25-27). The rationale behind STI, for example, was to allow for a transient re-expression of HIV-1 in latently infected cells. The goal was to render the cryptic virus visible to immune recognition and elimination. Another strategy was based on the administration of intermittent cycles of IL-2. This approach was based on phase II trials showing that treatment with IL-2 led to sustained and stable increases of peripheral CD4 T cell counts particularly in conjunction with HAART (28-30). However, recent results from two lengthy phase III trials (SIILCAT and ESPRIT) presented at CROI in Feb 2009 unequivocally demonstrated that HAART plus IL-2 offered no additional protection from death and AIDS-defining events when compared to HAART alone (see www.retroconference.org/2009). To date, STI and other immune-enhancing strategies have met with only limited success (26,27), presumably due to an insufficient body of basic knowledge relating to the complexity of HIV-1/host

interactions, especially as it relates to the beneficial vs. harmful effects of immune activation.

In order to develop strategies capable of suppressing the harmful effects of immune activation, we first need to identify the factors driving chronic stimulation in HIV-1-infected patients. Studies have repeatedly demonstrated that HIV-1 antigenic stimulation is a major force fuelling immune activation and that antiretroviral therapy, by decreasing viral load, can reduce immune activation (31,32). However, other recent studies have suggested that increased translocation of bacterial products from the lumen of the GIT (due to early HIV-1-related damage to epithelium barrier) into the circulation may also play a critical role in activation (22,23). Elimination of microbial-driven activation will depend, in large part, on whether the damage to the GIT is reversible and can be repaired. Furthermore the involvement of bacterial microbes suggests that activation of innate immune responses may play an important and pivotal role in mediating and amplifying the pathogenic immune activation syndrome that accompanies and at least partially drives HIV-1 disease evolution.

Most studies of HIV-1 and immune activation have focused on $CD4^+$ and $CD8^+$ lymphocytes. The reason for the disproportionate emphasis on T cells relates, in large measure, to the fact that $CD4^+$ T cells are the primary targets and the major site of virus production, and that progressive $CD4^+$ T cell depletion is a fundamental hallmark of HIV-1/AIDS (33). Alternatively, CD8+ T cells are responsible for HIV-1-specific CTL responses and contribute to the control of viral replication by releasing CCR5-binding chemokines and other soluble antiviral factors (34, 35). However, cells of the MP lineage also play a critical role both in the initial establishment of HIV-1 infection and throughout the course of HIV-1/AIDS. This is not surprising given the HIV-1 is a member of the lentivirus subfamily of retroviruses. Indeed, animals infected with lentirviruses demonstrate that cells of the MP system (including bone marrow precursor cells, monocytes and fully differentiated tissue macrophages) are often the primary target of

infection. For example, Maedi-Visna Virus (MVV), a virus that causes pulmonary disease in sheep, infects only macrophages and DC and is accompanied by a wasting syndrome similar to HIV-1 enteropathy and a brain disease resembling AIDS dementia (36,37).

Another reason that cells of the MP system have received less attention than T cell is that they are difficult to study by non-invasive techniques (38). Cells of this MP lineage exhibit a high level of heterogeneity, inter-donor variability and extreme plasticity. In addition, tissue macrophages are easily activated during the course of cell isolation (39, 40). *In vitro* surrogate models (cell lines, primary MDM) have proven valuable for unraveling the complexities of HIV-1 entry and replication in macrophages and for assessing the response of macrophages to antiretroviral therapy (38). However, there are no guarantees that data obtained from *in vitro* studies will be representative of events occurring at the patient level. Similarly, studies of SIV conducted in non-human primates may not be a true reflection of HIV-1 pathogenesis in humans. Thus, a multi-faceted approach involving both *in vitro* and *ex vivo* studies may provide the best chance for obtaining critical new information on the role of macrophages in HIV-1 pathogenesis.

This thesis has used a multi-pronged *in vitro* and patient-based approach to address questions relating to the relationships between HIV-1, macrophage activation/polarization and microbial translocation. Chapters 2, 3 and 4 of the thesis examine the role of macrophage polarization (M1 vs. M2a) in the regulation of viral replication and the establishment of productive vs. latent infection. To our knowledge this is one of the first studies to demonstrate that HIV-1 can be differentially modulated by cytokine-induced changes in the phenotypic and functional properties of primary MDM. Importantly, it was also observed that M1 activation appears to protect macrophages from infection, whereas M2a activation and polarization may drive the formation of viral reservoirs. M2a, but not M1 polarization, was also associated with an increased the capacity of macrophages to transmit HIV-1 to $CD4^+$ T cells. Based on these results, it seems reasonable to hypothesize that strategies aimed at suppressing replication and driving the host immune system toward

an M1 phenotype may be helpful in controlling HIV-1 in macrophages and in preventing macrophage-to-T cell transmission.

Chapters 5 and 6 describe *ex vivo* studies conducted on peripheral blood and gastrointestinal biopsies obtained from antiretroviral-naïve African patients with late-stage HIV-1 disease and no evidence of opportunistic co-infections such as tuberculosis, CMV or enteric pathogens. Results from Chapter 5 revealed that Africans, both HIV-1-infected and uninfected, have higher levels of immune activation than their North America and European counterparts. Monocyte activation was higher in infected compared to uninfected Africans and was associated with an increase in the plasma ratio of IL-10:IL-12 suggesting that, at least in this group of patients, HIV-1 infection was associated with a shift towards an alternative (M2) monocyte activation profile. Interestingly, some of these monocyte activation markers were positively correlated with HIV-1 viremia (CCL2, CD16) where as others (sCD14, TNF) showed a strong positive correlation with plasma LPS suggesting that HIV-1 and microbial translocation contribute differentially to the activation of monocytes. Thus, HAART, on its own, may not be sufficient to eliminate all aspects of monocyte activation and consequent disease progression.

Finally, Chapter 6 reveals that, similar to inflammatory bowel disease (IBD), HIV-1 infection is associated with increased recruitment of inflammatory macrophages into the *lamina propria* of the intestine and that these cells appear to play a bi-functional role in controlling viral replication and mediating bacterial translocation. Based on these findings, it seems reasonable to hypothesize that approaches aimed at decreasing inflammation and host responsiveness to bacterial products, and of promoting protection and repair of the mucosal barrier of the GIT, may help reduce microbe-driven activation. The data also suggests that approaches used to treat Crohn's disease, for example anti-TNF Ab, may also prove useful in the treatment of HIV-1. Alternative approaches aimed at limiting the recruitment of inflammatory monocytes to the GIT (and tissues such as the brain) may also prove effective in reducing HIV-1-associated disease pathogenesis.

In summary, the prevention and treatment of HIV-1/AIDS has proven to be a formidable task. Despite these daunting challenges, significant incremental progress has been made through intense effort and dedication. I truly believe that an improved understanding of monocyte-macrophages and innate immunity responses in HIV infection may point the way to future advances in therapeutic intervention.

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